linking oligonucleotide, a melting curve was performed. For the melting experiment, a slide was placed in a cuvette containing 1.5 mL of hybridization buffer, and an apparatus similar to that used in Example 2, part B, was used. The absorbance signal due to the nanoparticles (520 nm) was monitored at each degree as the temperature of the substrate was increased from 20°C to 80°C, with a hold time of 1 minute at each integral degree. The nanoparticle signal dramatically dropped when the temperature passed 52°C. See Figure 19B. A first derivative of the signal showed a melting temperature of 55°C, which corresponds with the temperature seen for the oligonucleotide-nanoparticle conjugates and linking oligonucleotides hybridized in solution. See Figure 19B.

Example 11: Assay of a Polyribonucleotide Using Nanoparticle-Oligonucleotide Conjugates as Probes

The previous Examples utilized oligo-deoxyribonucleotides as targets in the assays. The present example demonstrates that the nanoparticle-oligonucleotide conjugates can also be used as probes in assaying a polyribonucleotide. The experiment was carried out by adding 1 μL of a solution of poly(rA) (0.004 A_{260} Units) to 100 μL of gold nanoparticles (~ 10 nM in particles) conjugated to dT_{20} (a 20-mer oligonucleotide containing thymidylate residues) through a mercaptoalkyl linker at the 5'-terminus. The conjugation procedure was that described in Example 3. Following freezing in a Dry Ice/isopropyl alcohol bath, thawing at room temperature, and spotting on a C18 TLC plate as described in Example 4, a blue spot characteristic of aggregation of the nanoparticles by hybridization was observed. Control experiments carried out in absence of the target gave a pink spot, rather than a blue spot.

Example 12: Assay for Protective Antigen DNA Segment of Anthrax Using Nanoparticle-Oligonucleotide Conjugates

In many cases amplification of a double-stranded DNA target by PCR is needed to provided sufficient material for an assay. The present example demonstrates that the nanoparticle-oligonucleotide conjugates can be used to assay for a DNA strand in the presence of its complement (*i.e.*, assaying for a single strand after thermal

dehybridization of a double-stranded target) and can recognize and specifically bind to an amplicon obtained from a PCR reaction.

A PCR solution containing a 141 base pair duplex amplicon of the Protective Antigen segment of Anthrax was provided by the Navy (sequence given in Figure 23). The assay for this amplicon was carried out by isolating the DNA from 100 μL of the PCR solution using a Qiaquick Nucleotide Removal Kit (Qiagen, Inc., Santa Clarita, CA) and the standard protocol for this kit, with the exception that elution of the DNA was effected with 10 mM phosphate buffer at pH 8.5, rather than with the buffer provided with the kit. The eluant was then evaporated to dryness on a Speed Vac (Savant). To this residue was added 5 μL of a master mix prepared by mixing equal volumes of each of two solutions of two different oligonucleotide-nanoparticle probes (see Figure 23). Each oligonucleotide-nanoparticle probe was prepared as described in Example 3. The solutions of the probes which were combined to form the master mix were prepared by adding 10 μL of 2 M NaCl and 5 μL of oligonucleotide blocker solution (50 pmoles of each Blocker oligonucleotide (see Figure 23 and below) in a 0.3 M NaCl, 10 mM phosphate, pH 7.0., solution) to 5 μL of full-strength (about 10 nM) nanoparticle-oligonucleotide solution. The amplicon-probe mixture was heated to 100°C for 3 minutes, then frozen in a DRY ICE/ethanol bath and allowed to come to room temperature. A small aliquot (2 μL) was spotted on a C18 TLC plate and allowed to dry. A strong blue spot indicative of hybridization was obtained.

Control tests carried out in the same manner in absence of the amplicon target DNA, in the absence of Probe 1, in the absence of Probe 2, or in the absence of the sodium chloride, were all negative, that is, gave a pink spot. Similarly a test carried out using probes 1 and 2 with a PCR amplicon derived from the Lethal Factor segment of Anthrax in place of the Protective Antigen Segment was negative (pink spot). These controls confirmed that both probes were essential, that salt conditions appropriate for hybridization were needed, and that the test was specific for the specified target sequence.

The oligonucleotide Blockers were added to inhibit binding of the second strand of the initial duplex target (*i.e.*, the strand complementary to the target strand) to regions of the target nucleic acid strand outside the segment that binds to the probes (see Figure 23 for sequences), since such binding interferes with binding of the nanoparticle oligonucleotide probes to the target strand. In this example, the Blocker oligonucleotides were complementary to the single-stranded target in regions not covered by the probes. An alternative scheme is to use blocker oligonucleotides that are complementary to the PCR complementary strand (the strand complementary to the target strand) outside the region that competes with the probe oligonucleotides.

Example 13: Direct assay of PCR Amplicons without isolation of the amplicons from the PCR solution

The procedure described in Example 12 involved separation of the PCR amplicon from the PCR solution before addition of the nanoparticle-oligonucleotide probes. For many purposes it would be desirable to be able to carry out the assay directly in the PCR solution without preliminary isolation of the polynucleotide products. A protocol for such an assay has been developed and is described below. This protocol has been performed successfully with several PCR products derived under standard conditions using a GeneAmp PCR Reagent Kit with Amplitaq DNA polymerase.

To 50 μL of the PCR sample solution, 5 μL of a mixture of two gold nanoparticle-oligonucleotide probes (0.008 A_{520} Units of each) was added, followed by addition of a solution made up from 1 μL of Blocker oligonucleotides (10 pmoles each), 5 μL of 5 M NaCl, and 2 μL of 150 mM $MgCl_2$. This mixture was heated for 2 minutes at 100°C to separate the strands of the duplex target, the tube was immersed directly in a cold bath (*e.g.*, Dry Ice/ethanol) for 2 minutes, then removed, and the solution allowed to thaw at room temperature (the freeze-thaw cycle facilitates hybridization of the probes with the target oligonucleotide). Finally, a few μL of the solution were spotted on a plate (*e.g.*, C18 RP TLC plate, a silica plate, a nylon membrane, etc.). As usual, blue color

signifies the presence of the targeted nucleic acid in the PCR solution; a pink color is negative for this target.

Example 14: Direct Recognition of Duplex Oligonucleotides Without Dehybridization, Using Assembly of Nanoparticle-Oligonucleotide Conjugates

In the previous Examples, double-stranded targets were dehybridized by heating to generate single strands which interacted with single-stranded oligonucleotide probes bound to nanoparticles. The present example demonstrates that in cases where triple-stranded complexes can form, double-stranded oligonucleotide sequences can be recognized by the nanoparticle probes without prior dehybridization of the target.

Tests were carried out with two different systems-- polyA:polyU and dA_{30}:dT_{30} -- by adding 1 :L of a solution containing 0.8 A_{260} Units of the target duplex in 100 µL of buffer (0.1 M NaCl, 10 mM phosphate, pH 7.0) to 100 µL of a colloidal solution of Au-sdT_{30} nanoparticle-oligonucleotide conjugate (~10 nM in particles; see Example 11) in 0.3 M NaCl, 10 mM phosphate buffer at pH 7.0. Subsequent quick freezing by immersing the tube in a Dry Ice/isopropyl alcohol bath and thawing by removing the tube from the bath and letting it stand at room temperature (22°C), followed by spotting 3 µL of the solution on a C18 TLC plate, afforded a blue spot characteristic of hybridization and aggregation of the nanoparticles.

The rationale for this test is that the nanoparticle probes (bearing pyrimidine oligonucleotides in this example) bind in a sequence specific manner at purine oligonucleotide/pyrimidine oligonucleotide sites along the duplex target. Since many binding sites are available on each double stranded entity, the binding leads to formation of an aggregate of nanoparticles. The results show that this assay, based on formation of triple-stranded complexes involving the nanoparticle probes, works both for oligoribo- and oligodeoxyribonucleotide double-stranded targets.

Example 15: Assay Employing Both Fluorescence And Calorimetric Detection

All hybridization experiments were performed in a 0.3 M NaCl, 10 mM phosphate, pH 7.0, buffer solution. AcetatePlus™ filtration membranes (0.45 μm) were purchased from Micron Separations Inc., Westboro, MA. Alkylamine-functionalized latex microspheres (3.1 μm) were purchased from Bangs Laboratories, Fishers IN. Fluorophore-labeled oligonucleotides functionalized with alkylamino groups at the 3'-terminus were synthesized using standard phosphoramidite chemistry (Eckstein, ed., in *Oligonucleotides and Analogues*, 1st ed., Oxford University, New York, N.Y. 1991) with an Amino-Modifier C7 CPG solid support (Glen Research) and a 5'-fluorescein phosphoramidite (6-FAM, Glen Research) on an Expedite 8909 synthesizer and were purified by reverse phase HPLC. They were attached to the amine-functionalized latex microspheres by means of diisothiocyanate coupling to yield a dithiourea linkage as described in Charreyre et al., *Langmuir*, 13, 3103-3110 (1997). Briefly, a DMF solution of a one thousand fold excess of 1,4-phenylene diisothiocyanate was added to an aqueous borate buffer solution (0.1 M, pH 9.3) of the amino-modified oligonucleotide. After several hours, the excess 1,4-phenylene diisothiocyanate was extracted with butanol and the aqueous solution lyophilized. The activated oligonucleotides were redissolved in borate buffer and reacted with the amino-functionalized latex microspheres in a carbonate buffer (0.1 M, pH 9.3, 1 M NaCl). After 12 hrs, the particles were isolated by centrifugation and washed three times with buffered saline solution (0.3 M NaCl, 10 mM phosphate pH 7.0). The 5'-oligonucleotide-modified gold nanoparticle probes were prepared as described in Example 3.

The target oligonucleotide (1-5 μl, 3 nM) was added to 3 μl of fluorophore-labeled oligonucleotide-modified latex microsphere probe solution (3.1 μm; 100 fM). After 5 minutes, 3 μl of the 5' oligonucleotide-modified gold nanoparticle probe solution (13 nm; 8 nM) were added to the solution containing the target and latex microsphere probes. Upon standing for an additional 10 minutes, the solution containing both probes and target was vacuum-filtered through the AcetatePlus membrane. The membrane retained the relatively large latex particles and allowed any non-hybridized gold

nanoparticle probes to pass through. In the presence of a sufficient concentration of target, the latex microspheres and the gold nanoparticles hybridized with the target, and a red spot was observed on the membrane (positive result). A control experiment was always carried out where the aliquot of solution containing the target oligonucleotide was replaced by an equal volume of water. In this case, a white spot was left on the membrane (negative result). For a 24-base-pair model system, using the unaided eye, 3 femtomoles of target oligonucleotide could be detected colorimetrically.

A double-stranded target oligonucleotide (1-5 μl, 20 nM), 3 μl of a solution of fluorophore-labeled- oligonucleotide-latex microspheres (3.1 μm; 100 fM) and 3 μl of a solution of 5'-oligonucleotide-gold nanoparticles (13 nm; 8 nM) were combined and heated to 100 °C for 3 minutes. Then, the solution was immediately frozen by immersing the reaction vessel containing it in a liquid N_2 bath for 3 minutes. This solution was then thawed at room temperature and filtered as described above. For a 24-base pair model system, using the unaided eye, 20 femtomoles of duplex target oligonucleotide could be detected colorimetrically.

When monitored by fluorescence, the detection method described above proved to be difficult due to background fluorescence from the membrane. This problem was overcome by "washing" the latex microspheres by centrifugation to remove excess gold nanoparticle probes before spotting an aliquot on a reverse-phase TLC plate. The hybridization experiments were performed as described above. After hybridization was effected between the probes and target, 10 μl of buffer were added to the solution, which was subsequently centrifuged at 10,000 x g for 2 minutes. The supernatant was removed, and 5 μl of buffer were added to help resuspend the precipitate. A 3 μl aliquot was then spotted on a reverse-phase TLC plate. For both single-stranded and duplex target oligonucleotides, 25 femtomoles could be detected colorimetrically by the naked eye. Fluorescent spots could be visualized by the naked eye with a hand-held UV-lamp until the target amount in the 3 μl aliquot used to form the spot was as low as 50 femtomoles. It is believed that optimization of this system will allow for detection of even lower

amounts of target nucleic acid.

Example 16: Assays Employing Silver Staining

DNA hybridization tests on oligonucleotide-modified substrates are commonly used to detect the presence of specific DNA sequences in solution. The developing promise of combinatorial DNA arrays for probing genetic information illustrates the importance of these heterogeneous sequence assays to future science. In most assays, the hybridization of fluorophore-labeled targets to surface-bound probes is monitored by fluorescence microscopy or densitometry. Although fluorescence detection is very sensitive, its use is limited by the expense of the experimental equipment and by background emissions from most common substrates. In addition, the selectivity of labeled oligonucleotide targets for perfectly complementary probes over those with single-base mismatches is poor, preventing the use of surface hybridization tests for detection of single nucleotide polymorphisms. A detection scheme which improved upon the simplicity, sensitivity and selectivity of fluorescent methods could allow the full potential of combinatorial sequence analysis to be realized. The present invention provides such improved detection schemes.

For instance, oligonucleotide-modified gold nanoparticles and unmodified DNA target could be hybridized to oligonucleotide probes attached to a glass substrate in a three-component sandwich assay (see Figures 25A-B). Note that the nanoparticles can either be individual ones (see Figure 25A) or "trees" of nanoparticles (see Figure 25B). The "trees" increase signal sensitivity as compared to the individual nanoparticles, and the hybridized gold nanoparticles "trees" often can be observed with the naked eye as dark areas on the glass substrate. When "trees" are not used, or to amplify the signal produced by the "trees," the hybridized gold nanoparticles can be treated with a silver staining solution. The "trees" accelerate the staining process, making detection of target nucleic acid faster as compared to individual nanoparticles.

The following is a description of one specific system (illustrated in Figure 25A).

Capture oligonucleotides (3' - $HS(CH_2)_3$ - A_{10}ATGCTCAACTCT; SEQ ID NO: 43) were immobilized on a glass substrate as described in Example 10. A target oligonucleotide (5' - TACGAGTTGAGAATCCTGAATGCG - 3', SEQ ID NO: 44, concentrations given below in Table 6 for each experiment) was hybridized with the capture oligonucleotides in 0.3 M NaCl, 10 mM phosphate buffer as described in Example 10. The substrate was rinsed twice with the same buffer solution and immersed in a solution containing gold nanoparticle probes functionalized with target-complementary DNA (5' - $HS(CH_2)_6A_{15}$CGCATTCAGGAT, SEQ ID NO: 45)(preparation described in Example 3) for 12 hours. Next, the substrate was rinsed copiously with 0.3 M $NaNO_3$ to remove Cl^-. The substrate was then developed with silver staining solution (1:1 mixture of Silver Enhancer Solutions A and B, Sigma Chemical Co., # S-5020 and # S-5145) for 3 minutes. Greyscale measurements were made by scanning the substrate on a flatbed scanner (normally used for scanning documents into a computer) linked to a computer loaded with software capable of calculating greyscale measurements (*e.g.*, Adobe Photoshop). The results are presented in Table 6 below.

TABLE 6

Target DNA Concentration	Mean Greyscale	Standard Deviation
10 nM	47.27	2.10
5 nM	53.45	0.94
2 nM	54.56	1.17
1 nM	59.98	1.82
500 pM	64.61	2.26
200 pM	90.06	3.71
100 pM	99.04	2.84
50 pM	135.20	7.49
20 pM	155.39	3.66
None (control)	168.16	10.03

Example 17: Assemblies Containing Quantum Dots

This example describes the immobilization of synthetic single-stranded DNA on semiconductor nanoparticle quantum dots (QDs). Native CdSe/ZnS core/shell QDs (~4 nm) are soluble only in organic media, making direct reaction with alkylthiol-terminated single-stranded DNA difficult. This problem was circumvented by first capping the QDs with 3-mercaptopropionic acid. The carboxylic acid group was then deprotonated with 4-(dimethylamino)pyridine, rendering the particles water soluble, and facilitating reaction of the QDs with either 3'-propylthiol- or 5'-hexylthiol-modified oligonucleotide sequences. After DNA modification, the particles were separated from unreacted DNA by dialysis. A "linker" DNA strand was then hybridized to surface-bound sequences, generating extended assemblies of nanoparticles. The QD assemblies, which were characterized by TEM, UV/Visible spectroscopy, and fluorescence spectroscopy, could be reversibly assembled by controlling the temperature of the solution. The temperature dependent UV-Vis spectra were obtained for the novel QD assemblies and composite aggregates formed between QDs and gold nanoparticles (~13 nm).

A. General Methods

Nanopure water (18.1 MΩ) prepared using a NANOpure ultrapure water purification system was employed throughout. Fluorescence spectra were obtained using a Perkin Elmer LS 50 B Luminescence Spectrometer. Melting analyses were performed using a HP 8453 diode array spectrophotometer equipped with a HP 9090a Peltier Temperature Controller. Centrifugation was carried out using either an Eppendorf 5415C centrifuge or a Beckman Avanti 30 centrifuge. TEM images were acquired using a Hitachi HF-2000 field emission TEM operating at 200 kV.

B. Preparation Of Oligonucleotide-QD Conjugates

Synthetic methodologies for semiconductor quantum dots (QDs) have improved greatly in recent years, and for some materials, most notably CdSe, monodisperse samples of pre-determined size can now be prepared with relative ease. Murray et al., *J.*

Am. Chem. Soc. 1993, *115*, 8706; Hines, et al., *J. Phys. Chem.* 1996, *100*, 468. As a result, the unique electronic and luminescent properties of these particles have been studied extensively (see, Alivisatos, *J. Phys. Chem.* 1996, *100*, 13226, and references therein; Klein et al., *Nature* 1997, 699; Kuno et al., *J. Chem. Phys.* 1997, *106*, 9869; Nirmal et al., *Nature* 1996, *383*, 802), potentially paving the way for QDs to be employed in diverse technologies, such as light-emitting diodes (Schlamp et al., *J. Appl. Phys.* 1997, *82*, 5837; Dabbousi et al., *Appl. Phys. Lett.* 1995, *66*. 1316) and as non-radioactive biological labels (Bruchez et al., *Science* 1998, *281*, 2013; Chan et al., *Science* 1998, *281*, 2016). However, many applications will require that the particles be arranged spatially on a surface or organized into three-dimensional materials (Vossmeyer et al., *J. Appl. Phys.* 1998, *84*, 3664). Moreover, the ability to organize one or more types of nanoparticles into superlattice structures (Murray et al., *Science* 1995, *270*, 1335) would allow for the construction of completely new types of hybrid materials with new and potentially interesting and useful properties.

DNA is the ideal synthon for programming the assembly of nanoscale building blocks into periodic two- and three-dimensional extended structures. The many attributes of DNA, which include ease of synthesis, extraordinary binding specificity, and virtually unlimited programmability by virtue of nucleotide sequence, can be exploited for the use of QD assembly.

The modification of QDs with DNA has proven to be more difficult than for gold nanoparticles. The common methods for preparing highly luminescent CdSe QDs yield materials that are coated with a mixture of trioctylphosphine oxide (TOPO) and trioctylphosphine (TOP). As a result, these QDs are soluble only in non-polar solvents, making them difficult to functionalize with highly charged DNA strands by direct reaction. This difficulty has been overcome by the method described below, which is the first successful modification of semiconductor nanoparticles with single-stranded DNA. It should be noted that others, in elegant studies, have looked at the interactions between QDs and duplex DNA, but these studies did not make use of the sequence specific

binding properties of DNA to direct the assembly of extended QD structures. Coffer et al., *Appl. Phys. Lett*, 1996, *69*, 3851; Mahtab et al., *J. Am. Chem. Soc.*, 1996, *118*, 7028.

Since the surface of CdSe/ZnS core/shell QDs binds organic thiols, it was desired to modify these semiconductor particles with alkylthiol-terminated DNA strands by a substitution reaction. The lack of water solubility of these QDs, though, hindered such an approach. Two different methods recently have been reported for making QDs water soluble, allowing for the immobilization of protein structures on the QD surfaces. One involves encapsulation of the core/shell structures with a silica layer (Bruchez et al., *Science* 1998, *281*, 2013), while the other utilizes mercaptoacetic acid both to stabilize the particles and provide water solubility (Chan et al., *Science* 1998, *281*, 2016). The procedure described in this example, which produces remarkably stable colloid under DNA hybridization conditions, utilizes 3-mercaptopropionic acid to passivate the QD surface.

An excess of 3-mercaptopropionic acid (0.10 mL, 1.15 mmol; Aldrich) was added by syringe to a suspension of ~20 mg of TOP/TOPO stabilized CdSe/ZnS QDs (prepared as described in Hines, et al., *J. Phys. Chem.* 1996, *100*, 468) in 1.0 mL of N,N,-dimethyl formamide (DMF; Aldrich) generating a clear, dark orange solution containing 3-mercaptopropionic acid functionalized QDs. The reaction occurred quickly. For subsequent reactions, excess 3-mercaptopropionic acid was not removed, and the particles were stored at room temperature in DMF.

However, to characterize the QDs, a portion of the sample was purified by removing unreacted 3-mercapto-propionic acid as follows. A 0.50 mL sample was centrifuged (4 hours at 30,000 rpm), and the supernatant was removed. The remaining solution was washed with ~0.3 mL of DMF and recentrifuged. This step was repeated two additional times before recording the FTIR spectrum. FTIR (polyethylene card, 3M):1710 cm^{-1} (s), 1472 cm^{-1} (m), 1278 cm^{-1} (w), 1189 cm^{-1} (m), 1045 cm^{-1} (w), 993 cm^{-1} (m), 946 cm^{-1} (w), 776 cm^{-1} (m), 671 cm^{-1} (m). Unlike the TOP/TOPO stabilized native QDs, the 3-mercaptopropionic acid modified QDs exhibited a characteristic ν_{CO} band at

1710 cm^{-1} for the surface bound propionic acid.

Although the 3-mercaptopropionic acid modified QDs were practically insoluble in water, their solubility could be significantly enhanced by deprotonating the surface bound mercaptopropionic acid sites with 4-(dimethylamino)pyridine (DMAP; Aldrich) as described in the next paragraph. The QDs then dispersed readily in water, producing orange solutions that were stable for up to a week at room temperature.

To attach oligonucleotides to QDs, 150 μL (optical density at 530 nm = 21.4) of a solution of the 3-mercaptopropionic acid functionalized particles in DMF were added to a solution of DMAP (8.0 mg, 0.065 mmol) in 0.4 mL of DMF. An orange precipitate was formed. It was separated by centrifugation (~30 seconds at 3000 rpm) and then dissolved in 1.0 mL of a solution of 3'propylthiol- or 5' hexylthiol-terminated oligonucleotides (1.0-2.0 ODs/mL; prepared as described in Example 1; sequences given below). Precipitate (dissolved in water) was characterized by IR spectroscopy (polyethylene card, 3M). IR (cm^{-1}): 1647 (m), 1559 (s), 1462 (m), 1214 (w), 719 (w), 478 (s). After standing for 12 hours, the oligonucleotide-containing solution was brought to 0.15 M NaCl, and the particles were aged for an additional 12 hours. The NaCl concentration was then raised to 0.3 M, and the mixture was allowed to stand for a further 24-40 hours before dialyzing against PBS (0.3 M NaCl, 10 mM phosphate buffer, pH 7, 0.01% sodium azide) using a 100 kDa membrane (Spectra/Por Cellulose Ester Membrane). The dialysis was carried out over a period of 48 hours, during which time the dialysis bath was refreshed three times.

Oligonucleotide-QD conjugates prepared in this manner displayed indefinite aqueous stability. Moreover, the colloid remained strongly fluorescent, with a sharp [full width at half maximum (FWHM) = 33 nm], symmetrical emission at 546 nm (indicative of a ~3.2 nm CdSe core; Murray et al., *J. Am. Chem. Soc.* 1993, *115*, 8706).

Two different oligonucleotide-QD conjugates were prepared by this protocol and stored in PBS. One was modified with a 22mer, comprised of a propylthiol functionality at the 3'-end, a 12mer capture sequence, and an intervening 10 base (all A) spacer: 5'-

TCTCAACTCGTAA$_{10}$-(CH$_2$)$_3$-SH [SEQ ID NO: 46]. The other employed a 5'-hexylthiol-terminated sequence, also with a 10 base (all A) spacer, and a 12mer capture sequence which was non-complementary with the 3'-propylthiol sequence: 5'-SH-(CH$_2$)$_6$-A$_{10}$CGCATTCAGGAT-3' [SEQ ID NO: 47].

C. Preparation Of QD Assemblies

When approximately equal quantities of these two oligonucleotides (200μL, OD$_{530}$=0.224 and 0.206, respectively) were mixed and then combined with 6μL (60 pmol) of a solution of a complementary linking 24mer sequence (5'-TACGAGTTGAGAATCCT-GAATGCG-3', SEQ ID NO: 48), QD assemblies formed within 20-30 minutes at room temperature, Figure 26. Faster linking took place when the mixture was frozen (-78° C) and then allowed to warm slowly to room temperature.

The clusters generated were not large enough to settle out of solution. However, they could be separated by centrifugation at relatively low speeds (10,000 RPM for 10 min), as compared with the unlinked particles (30,000 RPM for 2-3 hours).

The decrease in fluorescence upon hybridization was determined by integration of the fluorescence signal (320 nm excitation wavelength) from 475 nm to 625 nm of 4 pairs of samples. Each pair was prepared in the following manner. A solution of of 3' propylthiol-terminated DNA-modified particles (30 μL, optical density at 530 nm = 0.224) was combined with a solution of 5' hexylthiol-terminated DNA-modified QDs (30 μL, optical density at 530 nm = 0.206) in an Eppendorf centrifuge tube, and then diluted with 140 μL of PBS. The mixture was then split into two equal portions, and complementary "linker" DNA (3 μL, 30 pmol) was added to one, while non-complementary "linker" DNA (5'-CTACTTAGATCCGAGTGCCCACAT-3', SEQ ID NO: 49) (3 μL, 30 pmol) was added to the other. All eight of the samples were then frozen in a dry ice/acetone bath (-78°C), after which they were removed from the bath and allowed to warm slowly to room temperature. To estimate the change in fluorescence efficiency upon hybridization, the fluorescence intensities of the "target" (complementary "linker") samples were adjusted to account for the difference in

absorbance at 320 nm from the corresponding control samples, which contained non-complementary "linker".

The results showed that hybridization of QD/QD assemblies was accompanied by a decrease in integrated fluorescence intensity by an average of 26.4 ± 6.1%, and a ~2 nm red shift of the emission maximum, presumably due to cooperative effects between QDs, Figure 27A. Interestingly, Bawendi, *et al.* noticed a similar, albeit slightly larger, red shift when comparing the fluorescence of close-packed QDs and widely separated dots isolated in a frozen matrix (Murray et al., *Science* 1995, *270*, 1335). These changes in the fluorescence spectra may be an indication of excimer formation between QDs, but the exact nature of such a complex is still largely a matter of speculation. As expected, no aggregation was observed when the "linker" was missing or not complementary, or when either one of the two types of particles was absent.

The "melting" behavior of the DNA was monitored by observing the UV-Vis spectra of the aggregates as a function of temperature. For this "melting" analysis, the precipitate containing the QD/QD assemblies was centrifuged at 10,000 rpm for 10 minutes, washed with 7 μL of PBS, recentrifuged, and suspended in 0.7 mL of PBS. The UV/Visible spectroscopic signature of the assemblies was recorded at two degree intervals as the temperature was increased from 25°C to 75°C, with a holding time of 1 minute prior to each measurement. The mixture was stirred at a rate of 500 rpm to ensure homogeneity throughout the experiment. Temperature vs extinction profiles were then compiled from the extinction at 600 nm. The first derivative of these profiles was used to determine the "melting" temperatures.

The results, Figure 27B (T_m = 57°C), demonstrated conclusively that DNA had been immobilized on the QD surfaces and that hybridization was responsible for the assembly process. The transition also was extremely sharp when compared with DNA alone (FWHM of the respective first derivatives: 4°C vs 9°C), which is consistent with the formation of an aggregate structure with multiple DNA links per particle. An increase in extinction was observed upon denaturation, most likely because of a screening

effect whereby particles in the interiors of the assemblies are prevented from absorbing light by the surrounding QDs.

D. Preparation Of QD/Gold Assemblies

With DNA-functionalized QDs in hand, the assembly of hybrid assemblies made from multiple types of nanoparticle building blocks became feasible. To prepare these hybrid assemblies, a solution of ~17 nM 3'-hexylthiol-modified 13 nm gold nanoparticles (30 μL, ~5 fmol; prepared as described in Example 3) was mixed with a solution of 5'-hexylthiol-terminated DNA-modified QDs (15 μL, optical density at 530 nm = 0.206) in an Eppendorf centrifuge tube. "Linker" DNA (5 μL, 50 pmol) was added, and the mixture cooled to -78°C, and then allowed to warm slowly to room temperature, generating a reddish-purple precipitate. No aggregation behavior was observed unless both types of particles and a complementary target were present. After centrifugation (1 min at 3,000 rpm) and removal of the supernatant, the precipitate was washed with 100 μL of PBS and recentrifuged.

For "melting" analysis, the washed precipitate was suspended in 0.7 mL of PBS. UV-Vis spectroscopy was used to follow the changes in the surface plasmon resonance of the gold nanoparticles, so temperature vs. extinction profiles were compiled at 525 nm. Using the surface plasmon resonance of the gold nanoparticles provides a much more sensitive probe with which to monitor hybridization than does the UV-Vis spectroscopic signature of the QDs alone. Therefore, a "melting" experiment can be performed on a much smaller sample (~10% of the QD solution is needed), although the intensity of the plasmon band obscures the UV/Vis signal from the QDs. Similar to the pure QD system described above, a sharp (FWHM of the first derivative = 4.5°C) melting transition occurred at 58°C (see Figure 27D).

High resolution TEM images of these assemblies showed a network of gold nanoparticles interconnected by multiple QDs, Figure 27C. The QDs, which have a much lower contrast in the TEM image than gold nanoparticles, can be identified by their lattice fringes. They are just barely resolvable with the high resolution TEM, but clearly

indicate the periodic structure of these composite assemblies and the role that DNA plays in forming them.

E. Summary

The results described in this example definitively establish that the immobilization of DNA onto QD surfaces has been achieved and that these particles can now be used in combination with DNA under hybridization conditions. Using DNA-functionalized QDs, the first DNA-directed formation of QD and mixed gold/QD nanoparticle structures has been demonstrated. The successful modification of semiconductor QDs with DNA has significant implications for materials research, and the door is now open for more extensive inquiries into the luminescent, electronic, and chemical properties of these unique building blocks as they are incorporated into new and functional *multi-component* nanostructures and nanoscale materials.

Example 18: Methods of Synthesizing Oligonucleotide-Nanoparticle Conjugates And The Conjugates Produced By The Methods

A. General Methods

$HAuCl_4 \cdot 3H_2O$ and trisodium citrate were purchased from Aldrich chemical company, Milwaukee, WI. Gold wire, 99.999% pure, and titanium wire were purchased from Goldsmith Inc., Evanston, IL. Silicon wafers (100) with a 1 micron thick oxide layer were purchased from Silicon Quest International, Santa Clara, CA. 5'-thiol-modifier C6-phosphoramidite reagent, 3'-propylthiol modifier CPG, fluorescein phosphoramidite, and other reagents required for oligonucleotide synthesis were purchased from Glen Research, Sterling, VA. All oligonucleotides were prepared using an automated DNA synthesizer (Expedite) using standard phosphoramidite chemistry (Eckstein, F. *Oligonucleotides and Analogues*; 1st ed.; Oxford University Press, New York, 1991). Oligonucleotides containing only 5' hexylthiol modifications were prepared as described in Example 1. 5-(and 6)-carboxyfluorescein, succinimidyl ester was purchased from Molecular Probes, Eugene, OR. NAP-5 columns (Sephadex G-25

Medium, DNA grade) were purchased from Pharmacia Biotech. Nanopure H_2O (>18.0 MΩ), purified using a Barnstead NANOpure ultrapure water system, was used for all experiments. An Eppendorf 5415C or a Beckman Avanti 30 centrifuge was used for centrifugation of Au nanoparticle solutions. High Performance Liquid Chromatography (HPLC) was performed using a HP series 1100 HPLC.

B. Physical Measurements.

Electronic absorption spectra of the oligonucleotide and nanoparticle solutions were recorded using a Hewlett-Packard (HP) 8452a diode array spectrophotometer. Fluorescence spectroscopy was performed using a Perkin-Elmer LS50 fluorimeter. Transmission Electron Microscopy (TEM) was performed with a Hitachi 8100 Transmission Electron Microscope operating at 200 kV. A Thermo Jarrell Ash AtomScan 25 atomic emission spectrometer with an inductively coupled plasma (ICP) source was used to determine the atomic concentration of gold in the nanoparticle solutions (gold emission was monitored at 242.795 nm).

C. Synthesis and Purification of Fluorescein-Labeled Alkanethiol-Modified Oligonucleotides

Thiol-modified oligonucleotide strands containing either 12 or 32 bases, with 5' hexylthiol and 3' fluorescein moieties, were prepared. The sequence of the 12mer (S12F) was $HS(CH_2)_6$-5'-CGC-ATT-CAG-GAT-3'-$(CH_2)_6$-F [SEQ ID NO:50], and the 32mer (SA_{20}12F) contained the same 12mer sequence with the addition of a 20 dA spacer sequence to the 5' end [SEQ ID NO:51]. The thiol-modified oligonucleotides were prepared as described in Storhoff et al., *J. Am. Chem.Soc.* 120:1959-1964 (1998). An amino-modifier C7 CPG solid support was used in automated synthesis, and the 5' terminus was manually modified with hexylthiol phosphoramidite, as described previously. The 3' amino, 5' trityl-protected thiol modified oligonucleotides were purified by reverse-phase HPLC using an HP ODS Hypersil column (5 mm, 250 x 4 mm) with 0.03 M triethyl ammonium acetate (TEAA), pH 7 and a 1%/ minute gradient of 95% CH_3CN / 5% 0.03 M TEAA at a flow rate of 1 mL/min., while monitoring the UV signal

of DNA at 254 nm. The retention times of the 5'-S-trityl, 3' amino modified 12-base and 32-base oligonucleotides were 36 and 32 minutes respectively.

The lyophilized product was redispersed in 1 ml of 0.1 M Na_2CO_3 and, while stirring in the dark, 100 μL of 10 mg/ml succinimidyl ester of fluorescein (5,6 FAM-SE, Molecular Probes) in dry DMF was added over 1.5 hours according to the directions of the manufacturer (Molecular Probes literature). The solution was stirred at room temperature for an additional 15 hours, then precipitated from 100% ethanol at -20 °C. The precipitate was collected by centrifugation, dissolved in H_2O and the coupled product separated from unreacted amino-terminated oligonucleotide by ion-exchange HPLC. A Dionex Nucleopac PA-100 column (250 x 4 mm) was operated with 10 mM NaOH aqueous eluent and a 1% / minute gradient of 1 M NaCl/10mM NaOH at a flow rate of 0.8 mL/minute. Retention times of 5'-S-trityl, 3' fluorescein modified 12mer and 32mer were 50 and 49 minutes respectively. The oligonucleotide product was desalted by reverse-phase HPLC. Removal of the trityl protection group of the fluorescein-terminated, trilytoligonucleotide was performed using silver nitrate and dithiothreitol (DTT) as previously described (Storhoff et al., *J. Am. Chem.Soc.* 120:1959-1964 (1998)). The yield and purity of the oligonucleotides were assessed using the techniques previously described for alkylthiol oligonucleotides (Storhoff et al., *J. Am. Chem.Soc.* 120:1959-1964 (1998)). Oligonucleotides were used immediately after detritylation of the thiol group.

Thiol-modified oligonucleotides containing 32 bases, with 3' propylthiol and 5' fluorescein moieties ($HS(CH_2)_3$-3'-$(W)_{20}$-TAG-GAC-TTA-CGC-5'-$(CH_2)_6$-F, W= A or T) [SEQ ID NO:52] were synthesized on an automated synthesizer using 3' thiol modifier CPG. The 5' terminus of each oligonucleotide was coupled manually to fluorescein phosphoramidite (6-FAM, Glen Research). The modified oligonucleotides were purified by ion exchange HPLC (1% / min gradient of 1 M NaCl, 10 mM NaOH; retention time (Rt) ~ 48 min (W = T), Rt ~ 29 min (W = A)). After purification, the oligonucleotide solutions were desalted by reverse phase HPLC. The 3' thiol moieties were deprotected

with dithiothreitol by a procedure previously described (Storhoff et al., *J. Am. Chem.Soc.* 120:1959-1964 (1998)).

D. Synthesis and Purification of Fluorescein Labeled Oligonucleotides.

The fluorophore labeled complement (12'F) consisted of 12 bases 3'-GCG-TAA-GTC-CTA-5'-$(CH_2)_6$-F [SEQ ID NO:53] complementary to the 12mer sequence in S12F and SA_{20}12F. The oligonucleotide was synthesized using standard methods, and a syringe-based procedure, similar to the procedure reported above for the 5' alkylthiol modification, was used to couple fluorescein phosphoramidite (6-FAM, Glen Research) to the 5' end of the CPG-bound oligonucleotide. Purification was performed using reverse-phase HPLC as above. The fluorescein-labeled oligonucleotide had a retention time of 18 min. The fluorophore labeled complement, 3'12F (5'-ATC-CTG-AAT-GCG-F; [SEQ ID NO:54]) was prepared using an amino-modifier C7 CPG solid support for automated synthesis, followed by coupling of 5-(6)-carboxyfluorescein succinimidyl ester to the 3' amine using the procedure described above.

E. Preparation and Characterization of Gold Nanoparticles

Gold nanoparticles were prepared by citrate reduction of $HAuCl_4$ as described in Example 1. Transmission Electron Microscopy (TEM) performed with a Hitachi 8100 TEM was used to determine the size distribution of the resulting nanoparticles. At least 250 particles were sized from TEM negatives using graphics software (ImageTool). The average diameter of a typical particle preparation was 15.7 ± 1.2 nm. Assuming spherical nanoparticles and density equivalent to that of bulk gold (19.30 g/cm^3), an average molecular weight per particle was calculated (2.4×10^7 g/mol). The atomic gold concentration in a solution of gold nanoparticles was determined by ICP-AES (inductively coupled plasmon atomic emission spectroscopy). A gold atomic absorption standard solution (Aldrich) was used for calibration. Comparison of atomic gold concentration in the particle solution to the average particle volume obtained by TEM analysis yielded the molar concentration of gold particles in a given preparation, typically ~10 nM. By measuring the UV-vis absorbance of nanoparticle solutions at the surface

plasmon frequency (520 nm), the molar extinction coefficients (ε at 520 nm) were calculated for the particles, typically $4.2 \times 10^8\ M^{-1}\ cm^{-1}$ for 15.7 ± 1.2 nm diameter particles.

F. Preparation of Gold Thin Films.

Silicon wafers were cut into ~10 mm x 6 mm pieces and cleaned with piranha etch solution (4:1 concentrated H_2SO_4: 30% H_2O_2) for 30 min at 50 °C, then rinsed with copious amounts of water, followed by ethanol. (*Warning: piranha etch solution reacts violently with organic materials and should be handled with extreme caution.*) Metal was deposited at a rate of 0.2 nm/s using an Edwards Auto 306 evaporator (base pressure of 3×10^{-7} millibar) equipped with an Edwards FTM6 quartz crystal microbalance. The oxidized sides of the silicon were coated with a Ti adhesion layer of 5 nm, followed by 200 nm of gold.

G. Preparation of 5' Alkylthiol Oligonucleotide-Modified Gold Nanoparticles.

Gold nanoparticles were modified with fluorescein-alkylthiol oligonucleotides by adding freshly deprotected oligonucleotides to aqueous nanoparticle solution (particle concentration ~10 nM) to a final oligonucleotide concentration of 3 μM. After 24 hours, the solution was buffered at pH 7 (0.01 M phosphate), and NaCl solution was added (to final concentration of 0.1 M). The solution was allowed to 'age' under these conditions for an additional 40 hours. Excess reagents were then removed by centrifugation for 30 minutes at 14,000 rpm. Following removal of the supernatant, the red oily precipitate was washed twice with 0.3 M NaCl, 10 mM phosphate buffer, pH 7, solution (PBS) by successive centrifugation and redispersion, then finally redispersed in fresh buffer solution. Invariably, a small amount (~ 10% as determined by UV-vis spectroscopy) of nanoparticle is discarded with the supernatant during the washing procedure. Therefore, final nanoparticle concentrations were determined by TEM, ICP-AES, and UV-vis spectroscopy (see above). Extinction coefficients and particle size distributions did not change significantly as a result of the oligonucleotide modification.

H. Preparation of 5' Alkylthiol Oligonucleotide-Modified Gold Thin Films.

Silicon supported gold thin films were immersed in deposition solutions of deprotected alkylthiol modified oligonucleotides for equal times and buffer conditions as for the gold nanoparticles. Following oligonucleotide deposition, the films were rinsed extensively with 0.3 M PBS and stored in buffer solution. Gold was evaporated on one side only, leaving an unpassivated silicon/silicon oxide face. However, alkylthiol modified DNA did not adsorb appreciably to bare silicon oxide surfaces that were rinsed with PBS.

1. Quantitation of Alkylthiol-Oligonucleotides Loaded on Nanoparticles.

Mercaptoethanol (ME) was added (final concentration 12 mM) to fluorophore-labeled oligonucleotide modified nanoparticles or thin films in 0.3 M PBS, to displace the oligonucleotides. After 18 hours at room temperature with intermittent shaking, the solutions containing displaced oligonucleotides were separated from the gold by either centrifugation of the gold nanoparticles, or by removal of the gold thin film. Aliquots of the supernatant were diluted two-fold by addition of 0.3 M PBS, pH 7. Care was taken to keep the pH and ionic strength of the sample and calibration standard solutions the same for all measurements due to the sensitivity of the optical properties of fluorescein to these conditions (Zhao et al., *Spectrochimica Acta* 45A:1113-1116 (1989)). The fluorescence maxima (measured at 520 nm) were converted to molar concentrations of the fluorescein-alkylthiol modified oligonucleotide by interpolation from a standard linear calibration curve. Standard curves were prepared with known concentrations of fluorophore-labeled oligonucleotides using identical buffer and salt concentrations. Finally, the average number of oligonucleotides per particle was obtained by dividing the measured oligonucleotide molar concentration by the original Au nanoparticle concentration. Normalized surface coverage values were then calculated by dividing by the estimated particle surface area (assuming spherical particles) in the nanoparticle solution. The assumption of roundness is based on a calculated average roundness factor of 0.93. Roundness factor is computed as: (4 x pi x Area)/(perimeter x 2) taken from Baxes, Gregory, *Digital Image Processing*, p. 157 (1994).

J. Quantitation of the Hybridized Target Surface Density.

To determine the activity of attached oligonucleotides for hybridization, fluorophore-labeled oligonucleotides, which were complementary to the surface-bound oligonucleotides (12'F), were reacted with oligonucleotide modified surfaces (gold nanoparticles or thin films) under hybridization conditions (3 μM complementary oligonucleotide, 0.3 M PBS, pH 7, 24 hr). Non-hybridized oligonucleotides were removed from the gold by rinsing twice with buffered saline as described above. Then, the fluorophore-labeled oligonucleotides were dehybridized by addition of NaOH (final concentration ~ 50 mM, pH 11-12, 4 hr). Following separation of the solution containing the 12'F from the nanoparticle solutions by centrifugation, and neutralization of the solutions by addition of 1 M HCl, the concentrations of hybridized oligonucleotide and corresponding hybridized target surface density were determined by fluorescence spectroscopy.

K. Quantitation of Surface Coverage and Hybridization

Citrate stabilized gold nanoparticles were functionalized with 12mer fluorescein-modified alkylthiol DNA (HS-$(CH_2)_6$-5'-CGC-ATT-CAG-GAT-$(CH_2)_4$-F [SEQ ID NO:50]). Surface coverage studies were then performed by thoroughly rinsing away non-chemisorbed oligonucleotides, followed by removal of the fluorophore-labeled oligonucleotides from the gold surface, and quantitation of oligonucleotide concentration using fluorescence spectroscopy (as described above).

Removal of all the oligonucleotides from the gold surface and subsequent removal of gold nanoparticles from the solution is critical for obtaining accurate coverage data by fluorescence for several reasons. First, the fluorescence signal of labeled, surface bound DNA is efficiently quenched as a result of fluorescence resonance energy transfer (FRET) to the gold nanoparticle. Indeed, there is almost no measurable signal for fluorescein-modified oligonucleotides (12-32 nucleotide strands, sequences are given above) after they are immobilized on 15.7 ± 1.2 nm gold nanoparticles and residual oligonucleotide in solution is washed away. Second, the gold nanoparticles absorb a

significant amount of light between 200 nm and 530 nm, so their presence in solution during fluorescence measurements acts as a filter and diminishes the available excitation energy, as well as the intensity of emitted radiation. The gold surface plasmon band at 520 nm falls at the emission maximum of fluorescein.

Mercaptoethanol (ME) was used to rapidly displace the surface bound oligonucleotides by an exchange reaction. To examine the displacement kinetics, oligonucleotide-modified nanoparticles were exposed to ME (12 mM) for increasing periods of time prior to centrifugation and fluorescence measurements. The intensity of fluorescence associated with the solution free of nanoparticles can be used to determine how much oligonucleotide was released from the nanoparticles. The amount of oligonucleotide freed in exchange with ME increased until about 10 hours of exposure (Figure 29), which is indicative of complete oligonucleotide displacement. The displacement reaction was rapid, which is presumably due to the inability of the oligonucleotide film to block access of the ME to the gold surface (Biebuyck et al., *Langmuir* 9:1766 (1993)).

The average oligonucleotide surface coverage of alkylthiol-modified 12mer oligonucleotide (S12F) on gold nanoparticles was 34 ± 1 pmol/cm^2 (average of ten independent measurements of the sample.) For 15.7 ± 1.2 nm diameter particles, this corresponds to roughly 159 thiol-bound 12mer strands per gold particle. Despite slight particle diameter variation from batch to batch, the area-normalized surface coverages were similar for different nanoparticle preparations.

In order to verify that this method is useful for obtaining accurate oligonucleotide surface coverages, it was used to displace flourophore-labeled oligonucleotides from gold thin films, and the surface coverage data was compared with experiments aimed at getting similar information but with different techniques. In these experiments, gold thin films were subjected to a similar oligonucleotide modification and ME displacement procedure as the citrate stabilized gold nanoparticles (see above). The oligonucleotide displacement versus time curves for the gold thin films are very similar to those measured

for gold nanoparticles. This suggests a similar rate of displacement for the thin films, even though the typical surface coverage values measured for these films were somewhat lower than the oligonucleotide coverages on gold nanoparticles. Importantly, the oligonucleotide surface coverages on gold thin films measured by our technique (18 ± 3 pmol/cm^2) fall within the range of previously reported coverages on oligonucleotide thin films (10 pmol/cm^2 for a 25 base oligonucleotide on gold electrodes determined using electrochemistry or surface plasmon resonance spectroscopy (SPRS) (Steel et al., *Anal. Chem.* 70:4670-4677 (1998)). Differences in surface coverages are expected due to different oligonucleotide sequences and lengths, as well as film preparation methods.

The extent of hybridization of complementary fluorophore-labeled oligonucleotides (12F') to nanoparticles with surface-bound 12mer oligonucleotides was measured as described above. Briefly, S12F modified nanoparticles were exposed to 12F' at a concentration of 3 μM for 24 hours under hybridization conditions (0.3 M PBS, pH 7) and then rinsed extensively with buffer solution. Again, it was necessary to remove the hybridized strands from the gold before measuring fluorescence. This was accomplished by denaturing the duplex DNA in a high pH solution (NaOH, pH 11) followed by centrifugation. Hybridized 12'F amounted to 1.3 ± 0.2 pmol/cm^2 (approximately 6 duplexes per 15.7 nm particle; the average number of duplexes per particle was computed by multiplying the normalized hybridized surface coverage in pmol/cm^2 by the average particle surface area as found from size distributions measured by TEM.). In order to measure the extent of non-specific adsorption, S12F modified gold nanoparticles were exposed to fluorophore-labeled non-complementary 12 base oligonucleotides (12F') in 0.3 M PBS. After extensive rinsing (successive centrifugation/redispersion steps) and subsequent high pH treatment, the coverage of non-specifically adsorbed oligonucleotides on the nanoparticles was determined to be on the order of 0.1 pmol/cm^2. An analogous procedure was used to measure hybridization to S12F modified gold thin films in order to compare the hybridization results to reported values on gold electrodes. The degree of hybridization, 6 ± 2 pmol/cm^2, was consistent

with hybridization reported for mixed base 25mer on an gold electrode (2-6 pmol/cm^2) (Steel et al., *Anal. Chem.* 70:4670-4677 (1998)).

Surface coverages and hybridization values of the S12F/12F' system for both nanoparticles and thin films are summarized in Table 7. The most striking result is the low hybridization efficiency (~ 4 % of surface-bound strands on nanoparticles while 33 % of strands on thin films hybridize). Previous studies have shown similarly low hybridization for sufficiently densely packed oligonucleotide monolayers. This may reflect a low accessibility to incoming hybridizing strands, due to a combination of steric crowding of the bases, especially those near the gold surface, as well as electrostatic repulsive interactions.

L. Effect of Oligonucleotide Spacer on Surface Coverage and Hybridization.

Although the high coverage of the S12F oligonucleotide is advantageous in terms of nanoparticle stabilization, the low hybridization efficiency prompted us to devise a means of decreasing steric congestion around the hybridizing sequence. Oligonucleotides (32mer) were synthesized having a 20 dA spacer sequence inserted between the alkylthiol group and the original 12 base recognition sequence. This strategy was chosen based on the assumption that: 1) bases near the nanoparticle surface are sterically inaccessible because of weak interactions between the nitrogenous bases and the gold surface, as well as interstrand steric crowding, and 2) on a 15.7 nm diameter roughly spherical particle, 12mer sequences attached to the end of 20mer spacer units roughly perpendicular to the surface (Levicky et al., *J. Am. Chem. Soc.* 120:9787-9792 (1998)) will lead to a film with a greater free volume as compared with a film formed from the same 12mer directly bound to the surface.

While the surface density of single-stranded $SA_{20}12F$ strands (15 ± 4 pmol/cm^2) was lower than that of S12F (34 ± 1 pmol/cm^2), the particles modified with a 32-mer using the identical surface modification showed comparable stability compared to those modified with 12-mer. As anticipated, the hybridization efficiency of the $SA_{20}12F/12F'$ system (6.6 ± 0.2 pmol/cm^2, 44%) was increased to approximately 10 times that of the

original S12F/12F' system, Table 7.

M. Effect of Electrolyte Concentration During Oligonucleotide Attachment

In working with the S12F sequence a salt aging step was found to be crucial in obtaining stable oligonucleotide modified nanoparticles (see Example 3). The gold nanoparticles modified with S12F in pure water fused together irreversibly to form a black precipitate upon centrifugation, while those aged in salt resisted aggregation when centrifuged, even in high ionic strength solutions. It is proposed that the increased stability is due to higher oligonucleotide surface coverages which leads to greater steric and electrostatic protection. Using the $SA_{10}12F$ modified particles, the effect of electrolyte conditions on oligonucleotide surface loading was investigated. As shown in Table 8, final surface coverages for gold nanoparticles which were exposed to oligonucleotides in water for 48 hours are much lower (7.9 ± 0.2 pmol/cm^2) compared to those that were 'aged' in salt, or prepared by increasing the salt concentration gradually over the course of the final 24 hours of the experiment (see above).

It is important to note that gold nanoparticles as synthesized irreversibly agglomerate even in very low ionic strength media. Indeed, they are naturally incompatible with salts and especially polyanions such as oligonucleotides. This aging treatment is essential for preparing stable oligonucleotide particles. Therefore, the particles must be initially modified with alkylthiol oligonucleotides in water prior to gradually increasing the ionic strength. It is likely that oligonucleotides initially lie flat, bound through weak interactions of the nitrogenous bases with gold. A similar mode of interaction has been proposed for oligonucleotides on thin films (Herne et al., *J. Am. Chem. Soc.* 119:8916-8920 (1997)). However, the interaction between oligonucleotides and the positively charged nanoparticle surface (Weitz et al., *Surf. Sci.* 158:147-164 (1985)) is expected to be even stronger. In the aging step, the high ionic strength medium effectively screens charge repulsion between neighboring oligonucleotides, as well as, attraction between the polyanionic oligonucleotide and the positively charged gold surface. This allows more oligonucleotides to bind to the nanoparticle surface, thereby

increasing oligonucleotide surface coverage.

N. Effect of Oligonucleotide Spacer Sequence on Surface Coverage.

In order to examine how the sequence of the spacer affects oligonucleotide coverage on Au nanoparticles, fluorescein-modified 32-mer strands, with 20 dA and 20 dT spacers inserted between a 3' propylthiol and the fluorescein-labeled 12-mer sequence, were prepared. The most notable result of surface coverage and hybridization studies of nanoparticles modified with S3'T_{20}12F and S3'A_{20}12F is the greater surface coverage achieved with the 20 dT spacer (35 ± 1 pmol/cm^2), in comparison to the 20 dA spacer (24 ± 1 pmol/cm^2). The number of hybridized strands was comparable, although the percentage of surface bound strands which hybridized was lower for ST_{20}12mer nanoparticles (79 %) than the SA_{20}12 nanoparticles (~94%). These results suggest that dT rich oligonucleotide strands interact non-specifically with the nanoparticle surface to a lesser degree than dA rich oligonucleotide strands. Consequently, 20dT spacer segments may extend perpendicular from the gold surface, promoting higher surface coverages, while 20dA spacer segments block gold sites by lying flat on the particle surface.

O. Effect of Coadsorbed Diluent Oligonucleotides

In addition to efficient hybridization, another important property of oligonucleotide modified nanoparticles is the possibility of adjusting the total number of hybridization events. This is most readily accomplished by adjusting the surface density of recognition strands. Other researchers have used coadsorbed diluent alkylthiols such as mercaptohexanol with modified oligonucleotides on gold electrodes to control hybridization (Steel et al., *Anal. Chem.* 70:4670-4677 (1998); Herne et al., *J. Am. Chem. Soc.* 119:8916-8920 (1997)). However, the inherent low stability of unprotected gold nanoparticles poses serious constraints on the choice of diluent molecule. A thiol modified 20 dA sequence (SA_{20}) [SEQ ID NO:55] proved to be suitable in terms of maintaining particle stability in the high ionic strength buffers which are needed for hybridization and protecting the surface from non-specific adsorption.

Nanoparticles were modified using solutions containing different recognition

strand ($SA_{20}12F$) to diluent (SA_{20}) strand molar ratios. The resulting particles were analyzed by the fluorescence method described above to determine the $SA_{20}12F$ surface density, and then tested for hybridization efficiency with 12'F.

The $SA_{20}12F$ surface density increased linearly with respect to the proportion of $SA_{20}12F$ to SA_{20} in the deposition solution, Figure 30. This is an interesting result because it suggests that the ratio of $SA_{20}12F$ to SA_{20} attached to the nanoparticles reflects that of the solution. This result is in contrast to what is normally seen for mixtures of short chain alkyl or T-functionalized thiols, where solubility and chain length play a crucial role in adsorption kinetics (Bain et al., *J. Am. Chem. Soc.* 111:7155-7164 (1989); Bain et al., *J. Am. Chem. Soc.* 111:7164-7175 (1989)).

The amount of complementary 12F' oligonucleotide which hybridized to each different sample also increased linearly with increasing $SA_{20}12F$ surface coverage, Figure 31. The fact that this relationship is well defined indicates that it is possible to predict and control the extent of hybridization of the nanoparticle-oligonucleotide conjugates. This suggests that hybridization of 12F' becomes more difficult at higher $SA_{20}12F$ coverages, which is most likely a result of steric crowding and electrostatic repulsion between oligonucleotides.

P. Summary.

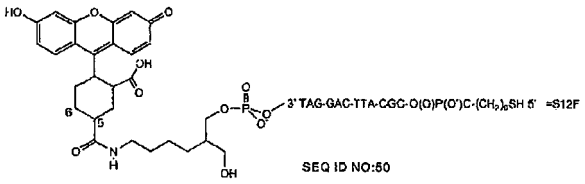
This study has shown that it is important to achieve a balance between oligonucleotide coverage high enough to stabilize the nanoparticles to which they are attached, yet low enough so that a high percentage of the strands are accessible for hybridization with oligonucleotides in solution. This has been achieved by adjusting salt conditions during oligonucleotide attachment to the nanoparticles to gain high oligonucleotide surface coverages, oligonucleotide spacer segments to reduce electrosteric interactions, and coadsorbed diluent strands to reproducibly control the average number of hybridization events for each nanoparticle. It has also been shown that the nature of the tether (spacer) sequence influences the number of oligonucleotide strands loaded onto gold nanoparticles. This work has important implications regarding

understanding interactions between oligonucleotides and nanoparticles, as well as optimizing the sensitivity of nanoparticle-oligonucleotide detection methods.

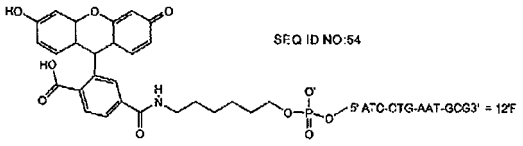
TABLE 7

Single strand surface coverage and corresponding hybridized surface coverages for gold thin films and gold nanoparticles. Comparison between S12F and $SA_{20}12F$ surface coverage and hybridization. Thiol modified oligonucleotides were attached to the gold from 3 μM aqueous solutions and aged in 0.1 M NaC^{+}. All hybridization studies were performed in 0.3 M PBS, pH 7.

Oligonucleotide Pair	Surface Coverage ($pmol/cm^2$)	Hybridization Coverage ($pmol/cm^2$)	% Hybridization Efficiency
	Au nanoparticles		
S12F/12F'	34 ± 1	1.3 ± 0.2	~ 4%
$SA_{20}12F/12F'$	15 ± 4	6.6 ± 0.2	~ 44%
	Au thin films		
S12F/12F'	18 ± 3	6 ± 2	~ 33%



SEQ ID NO:50



SEQ ID NO:54

TABLE 8

Effect of salt aging on surface coverage of $SA_{20}12F$ oligonucleotides to gold nanoparticles and hybridization to 12F'. All hybridization experiments were performed in 0.3 M PBS, pH 7.

Buffer conditions during adsorption of alkylthiol DNA	Surface Coverage (pmol/cm^2)	Hybridization Coverage (pmol/cm^2)	Hybridization Efficiency (%)
H_2O	7 9 ± 0.2	-- [a]	--
0.1 M NaCl, 10 mM phosphate	15 ± 4	6.6 ± 0.2	~44
1.0 M NaCl, 10 mM phosphate	20 ± 2	6.6 ± 0.2	~33

[a] Reliable values for these experiments could not be obtained due to a small amount of particle aggregation which occurred after centrifugation.

TABLE 9

Effect of oligonucleotide spacer sequence on surface coverage and hybridization efficiency.

Oligonucleotide Pair	Surface Coverage (pmol/cm^2)	Hybridization Coverage (pmol/cm^2)	Hybridization Efficiency (%)
S3'A_{20}12F / 3''2F	24 ± 1	9 ± 2	~38
S3'T_{20}12F / 3'12F	35 ± 1	12 ± 1	~34

S3'A_{20}12F / S3'T_{20}12F = $HS(CH_2)_6$-3'-W_{20}-TAG-GAC-TTA-CGC-5'-$(CH_2)_6$-F [SEQ ID NO:52]
3'12F = 5'-ATC-CTG-AAT-GCG-F [SEQ ID NO:54]

Example 19: Gene Chip Assay

An ultraselective and ultrasensitive method for analyzing combinatorial DNA arrays using oligonucleotide-functionalized gold nanoparticles is described in this example. An unusually narrow temperature range for thermal dissociation of nanoparticle-target complexes permits the discrimination of a given oligonucleotide sequence from targets with single nucleotide mismatches with extraordinary selectivity. In addition, when coupled with signal amplification method based on nanoparticle-catalyzed reduction of silver(I), the sensitivity of this nanoparticle array detection system

exceeds that of the analogous, conventional fluorophore system by two orders of magnitude.

Sequence-selective DNA detection has become increasingly important as scientists unravel the genetic basis of disease and use this new information to improve medical diagnosis and treatment. Commonly used heterogeneous DNA sequence detection systems, such as Southern blots and combinatorial DNA chips, rely on the specific hybridization of surface-bound, single-strand capture oligonucleotides complementary to target DNAs. Both the specificity and sensitivity of these assays are dependent upon the dissociation properties of capture strands hybridized to perfectly-matched and mismatched targets. As described below, it has surprisingly been discovered that a single type of nanoparticles hybridized to a substrate exhibits a melting profile that is substantially sharper than both the analogous fluorophore-based system and unlabeled DNA. Moreover, the melting temperature for the nanoparticle duplex is 11 degrees higher than for the analogous fluorophore system with identical sequences. These two observations, combined with the development of a quantitative signal amplification method based upon nanoparticle catalyzed reduction of silver(I), have allowed the development of a new chip-based detection system for DNA that has single-base mismatch selectivity and a sensitivity that is two orders of magnitude more sensitive than the conventional analogous fluorescence-based assays.

Gold nanoparticles (13 nm diameter) having oligonucleotide attached to them prepared as described in Example 3 were used to indicate the presence of a particular DNA sequence hybridized to a transparent substrate in a three-component sandwich assay format (see Figure 32). In a typical experiment, a substrate was fabricated by functionalizing a float glass microscope slide (Fisher Scientific) with amine-modified probe oligonucleotides as described in Example 10. This method was used to generate slides functionalized with a single type of oligonucleotides over their entire surface or in arrays of multiple types of oligonucleotides spotted with a commercial microarrayer. Nanoparticles having indicator oligonucleotides attached to them and synthetic 30-mer

oligonucleotide targets (based on the anthrax protective antigen sequence) were then cohybridized to these substrates (see Figure 32). Therefore, the presence of nanoparticles at the surface indicated the detection of a particular 30-base sequence. At high target concentrations (≥ 1 nM), the high density of hybridized nanoparticles on the surface made the surface appear light pink (see Figure 33). At lower target concentrations, attached nanoparticles could not be visualized with the naked eye (although they could be imaged by field-emission scanning electron microscopy). In order to facilitate the visualization of nanoparticles hybridized to the substrate surface, a signal amplification method in which silver ions are catalytically reduced by hydroquinone to form silver metal on the slide surface was employed. Although this method has been used for enlargement of protein- and antibody-conjugated gold nanoparticles in histochemical microscopy studies (Hacker, in *Colloidal Gold: Principles, Methods, and Applications*, M. A. Hayat, Ed. (Academic Press, San Diego, 1989), vol. 1, chap. 10; Zehbe et al., *Am. J. Pathol.* 150, 1553 (1997)) its use in quantitative DNA hybridization assays is novel (Tomlinson et al., *Anal. Biochem.*, 171:217 (1988)). Not only did this method allow very low surface coverages of nanoparticle probes to be visualized by a simple flatbed scanner or the naked eye (Figure 33), it also permitted quantification of target hybridization based on the optical density of the stained area (Figure 34). Significantly, in the absence of the target, or in the presence of noncomplementary target, no staining of the surface was observed, demonstrating that neither nonspecific binding of nanoparticles to the surface, nor nonspecific silver staining, occurs. This result is an extraordinary feature of these nanoparticle-oligonucleotide conjugates which enables ultra-sensitive and -selective detection of nucleic acids.

It has been determined that the unique hybridization properties of oligonucleotide-functionalized nanoparticles of the present invention can be further used to improve the selectivity of combinatorial oligonucleotide arrays (or "gene chips") (Fodor, *Science* 277, 393 (1997)). The relative ratio of target hybridized to different elements of an oligonucleotide array will determine the accuracy of the array in determining the target

sequence; this ratio is dependent upon the hybridization properties of the duplex formed between different capture strands and the DNA target. Remarkably, these hybridization properties are dramatically improved by the use of nanoparticle labels instead of fluorophore labels. As shown in Figure 35, the dehybridization of nanoparticle-labeled targets from surface-bound capture strands was much more sensitive to temperature than that of fluorophore-labeled targets with identical sequences. While the fluorophore-labeled targets dehybridized from surface capture strands over a very broad temperature range (first derivative FWHM = 16 °C), identical nanoparticle-labeled targets melted much more sharply (first derivative FWHM = 3 °C). It was anticipated that these sharpened dissociation profiles would improve the stringency of chip-based sequence analysis, which is usually effected by a post-hybridization stringency wash. Indeed, the ratio of target hybridized to complementary surface probes to that hybridized to mismatched probes after a stringency wash at a specific temperature (represented by the vertical lines in Figure 35) is much higher with nanoparticle labels than fluorophore labels. This should translate to higher selectivity in chip detection formats. In addition, nanoparticle labels should increase array sensitivity by raising the melting temperature (T_m) of surface duplexes, which lowers the critical concentration below which duplexes spontaneously melt at room temperature.

In order to evaluate the effectiveness of nanoparticles as colorimetric indicators for oligonucleotide arrays, test chips were probed with a synthetic target and labeled with both fluorophore and nanoparticle indicators. The test arrays and oligonucleotide target were fabricated according to published protocols (Guo et al., *Nucl. Acids Res.*, 22:5456 (1994); arrays of 175 μm diameter spots separated by 375 :m were patterned using a Genetic Microsystems 417 Microarrayer). Arrays contained four elements corresponding to the each of the four possible nucleotides (N) at position 8 of the target (see Figure 32). The synthetic target and either fluorescent-labeled or nanoparticle-labeled probes were hybridized stepwise to arrays in hybridization buffer, and each step was followed with a stringency buffer wash at 35 °C. First, 20 μL of a 1 nM solution of synthetic target in 2

X PBS (0.3 M NaCl, 10 mM NaH_2PO_4/Na_2HPO_4 buffer, pH 7) was hybridized to the array for 4 hours at room temperature in a hybridization chamber (Grace Bio-Labs Cover Well PC20), and then washed at 35°C with clean 2 X PBS buffer. Next, 20 μL of a 100 pM solution of oligonucleotide-functionalized gold nanoparticles in 2 X PBS was hybridized to the array for 4 hours at room temperature in a fresh hybridization chamber. The array was washed at 35°C with clean 2 X PBS, then twice with 2 X PBN (0.3 M $NaNO_3$, 10 mM NaH_2PO_4/Na_2HPO_4 buffer, pH 7). Then, the nanoparticle arrays were immersed in a silver amplification solution (Sigma Chemical, Silver Enhancer Solution) for 5 min and washed with water. Silver amplification darkened the array elements considerably, and 200 μm diameter elements could be easily imaged with a flatbed scanner or even the naked eye.

Arrays challenged with the model target and nanoparticle-labeled probes and stained with the silver solution clearly exhibited highly selective hybridization to complementary array elements (Figure 36A). Redundant spots of the same capture sequence showed reproducible and consistent hybridization signal. No background adsorption by nanoparticles or silver stain was observed; the image greyscale value reported by the flatbed scanner is the same as that observed for a clear microscope slide. The darker spots corresponding to adenine at position 8 (N=A) indicate that oligonucleotide target hybridized preferentially to perfectly complementary capture strands over mismatched ones, by a greater than 3:1 ratio. In addition, integrated greyscale values for each set of spots follows the predicted stability of the Watson-Crick base pairs, A:T > G:T > C:T > T:T (Allawi et al., *Biochemistry* 36, 10581, (1988)). Normally, G:T mismatches are particularly difficult to discriminate from A:T complements (Saiki et al., in *Mutation Detection*, Cotton et al., eds. (Oxford University Press, Oxford, 1998), chap. 7; S. Ikuta et al., *Nucl. Acids Res.* 15, 797 (1987)), and the distinction of these two array elements demonstrates the remarkable resolving power of nanoparticle labels in single nucleotide mismatch detection. The selectivity of the nanoparticle-based arrays was higher than that of the fluorophore-indicated arrays, Figure

36B; fluorophore labels provided only 2:1 selectivity for adenine at position 8.

The assays utilizing nanoparticle-labeled probes were significantly more sensitive than those utilizing fluorophore-labeled probes. Hybridization signal could be resolved at the N=A elements at target concentrations as low as 50 fM (or, for a hybridization chamber containing 20 μL of solution, 1×10^6 total copies); this represents a dramatic increase in sensitivity over common Cy3/Cy5 fluorophore-labeled arrays, for which ~ 1 pM or greater target concentrations are typically required. The higher melting temperatures observed for nanoparticle-target complexes immobilized on surfaces undoubtedly contribute to array sensitivity. The greater stability of the probe/target/surface-oligonucleotide complex in the case of the nanoparticle system as compared with the fluorophore system presumably results in less target and probe lost during washing steps.

Colorimetric, nanoparticle labeling of combinatorial oligonucleotide arrays will be useful in applications such as single nucleotide polymorphism analysis, where single mismatch resolution, sensitivity, cost and ease of use are important factors. Moreover, the sensitivity of this system, which has yet to be totally optimized, points toward a potential method for detecting oligonucleotide targets without the need for target amplification schemes such as polymerase chain reaction.

Example 20: Nanoparticle Structures

The reversible assembly of supramolecular layered gold nanoparticle structures onto glass supports, mediated by hybridized DNA linkers, is described. Layers of oligonucleotide-functionalized nanoparticles were successively attached to oligonucleotide-functionalized glass substrates in the presence of a complementary DNA linker. The unique recognition properties of DNA allow the nanoparticle structures to be assembled selectively in the presence of the complementary linker. In addition, the structures can be assembled and disassembled in response to external stimuli which mediate hybridization of the linking duplex DNA, including solution temperature, pH,

and ionic strength. In addition to offering a very selective and controlled way of building nanoparticle based architectures on a solid support, this system allows one to study the factors that influence both the optical and melting properties of nanoparticle network structures linked with DNA.

Others have demonstrated how bifunctional organic molecules (Gittins et al., *Adv. Mater.* 11:737 (1999); Brust et al., *Langmuir* 14:5425 (1998); Bright et al., *Langmuir* 14:5695 (1998); Grabar et al., *J. Am. Chem. Soc.* 118:1148 (1996); Freeman et al., *Science* 267:1629 (1995); Schmid et al., *Angew. Chem. Int. Ed. Engl.* 39:181 (2000); Marinakos et al., *Chem. Mater.* 10:1214 (1998)) or polyelectrolytes (Storhoff et al., *J. Am. Chem. Soc.* 120:1959 (1998); Storhoff et al., *J. Cluster Sci.* 8:179 (1997); Elghanian et al., *Science* 277:1078 (1997); Mirkin et al., *Nature* 382:607 (1996)) can be used to controllably construct mono- and multilayered nanoparticle materials off of planar substrates. The attractive feature of using DNA as a nanoparticle interconnect is that one can synthetically program interparticle distances, particle periodicities, and particle compositions through choice of DNA sequence. Moreover, one can utilize the reversible binding properties of oligonucleotides to ensure the formation of thermodynamic rather than kinetic structures. In addition to providing a new and powerful method for controlling the growth of nanoparticle-based architectures from solid substrates, this strategy also allows one to evaluate the relationship between nanoparticle aggregate size and both melting and optical properties of aggregate DNA-interlinked structures. An understanding of these two physical parameters and their relationship to materials architecture is essential for utilizing nanoparticle network materials, especially in the area of biodetection.

The oligonucleotide-functionalized, 13-nm-diameter gold nanoparticles used to construct the multilayer assemblies were prepared as described in Examples 1 and 3. The nanoparticles had 5'-hexanethiol-capped oligonucleotide 1 (5'-HS(CH_2)$_6$O(PO_4^-)O-CGCATTCAGGAT-3' [SEQ ID NO:50]) and 3'-propanethiol-capped oligonucleotide 2 (3'-HS(CH_2)$_3$O(PO_4^-)O-ATGCTCAACTCT-5' [SEQ ID NO:59]) attached to them to

yield nanoparticles *a* and *b*, respectively (see Figure 37). Glass slides were functionalized with 12-mer oligonucleotide 2 as described in Example 10. To build nanoparticle layers, the substrates were first immersed in a 10 nM solution of 24-mer linker 3 (5'-TACGAGTTGAGAATCCTGAATGCG-3' [SEQ ID NO:60]) and allowed to hybridize with it for 4 hours at room temperature (see Figure 37). The substrates were washed with clean buffer solution, and then hybridized with a 2 nM solution of particle *a* for 4 hours at room temperature to attach the first nanoparticle layer. A second nanoparticle layer could be attached to the first one by similarly exposing the surface to solutions of linker 3 and nanoparticle *b*. These hybridization steps could be repeated to attach multiple, alternating layers of nanoparticles *a* and *b*, each layer connected to the previous one by linker 3. In the absence of linker, or in the presence of noncomplementary oligonucleotide, no hybridization of nanoparticles to the surface was observed. In addition, multilayer assembly was only observed under conditions which promoted the hybridization of the DNA linkers: neutral pH, moderate salt concentration (> 0.05 M NaCl), and a temperature below the duplex melting temperature (T_m).

Each hybridized nanoparticle layer imparted a deeper red color to the substrate, and after ten hybridized layers, the supporting glass slide appeared reflective and gold in color. Transmission UV-vis spectroscopy of the substrate was used to monitor the successive hybridization of nanoparticle layers to the surface, Figure 38A. The low absorbance of the initial nanoparticle layer suggests that it seeded the formation of further layers, which showed a near linear increase in the intensity of the plasmon band with each additional layer (for each successive nanoparticle layer formation, no additional absorbance was observed on exposure for longer times or to higher concentrations of either linker 3 or nanoparticle solution). The linearity of the absorbance increase after the generation of the initial nanoparticle layer indicates that the surface was saturated with hybridized nanoparticles with each successive application, Figure 38B. This is supported by field-emission scanning electron microscope (FE-SEM) images of one (Figure 39A) and two (Figure 39B) nanoparticle layers on a surface, which show low nanoparticle

coverage with one layer, but near complete coverage with two layers. The λ_{max} of the plasmon band for the multilayer assemblies shifts no more than 10 nm, even after 5 layers. The *direction* of this shift is consistent with other experimental (Grabar et al., *J. Am. Chem. Soc.* 118:1148 (1996)) and theoretical (Quinten et al., *Surf. Sci.* 172:557 (1986); Yang et al., *J. Chem. Phys.* 103:869 (1995)) treatments of gold nanoparticle aggregates. However, the *magnitude* of the shift is small compared to that previously observed for suspensions of oligonucleotide-linked gold nanoparticle networks, which show $\lambda_{max} > 570$ nm (see previous examples). This suggests that many more linked nanoparticles --- perhaps hundreds or thousands --- are required to produce the dramatic color change from red to blue observed for gold nanoparticle-based oligonucleotide probes. (Storhoff et al., *J. Am. Chem. Soc.* 120:1959 (1998); Storhoff et al., *J. Cluster Sci.* 8:179 (1997); Elghanian et al., *Science* 277:1078 (1997); Mirkin et al., *Nature* 382:607 (1996).). Surface plasmon shifts for aggregated gold nanoparticles have been shown to be highly dependent on interparticle distance (Quinten et al., *Surf. Sci.* 172:557 (1986); Storhoff et al., *J. Am. Chem. Soc.*, in press), and the large distances provided by oligonucleotide linkers (8.2 nm for this system)) significantly reduce the progressive effect of nanoparticle aggregation on the gold surface plasmon band.

The dissociation properties of the assembled nanoparticle multilayers were highly dependent upon the number of layers. When the multilayer-coated substrates were suspended in buffer solution and the temperature raised above the T_m of the linking oligonucleotides (53°C), the nanoparticles dissociated into solution, leaving behind a colorless glass surface. Increasing or decreasing the pH (>11 or <3) or decreasing the salt concentration of the buffer suspension (below ~0.01 M NaCl) also dissociated the nanoparticles by dehybridizing the linking DNA. The multilayer assembly was fully reversible, and nanoparticles could be hybridized to, and dehybridized from, the glass substrates (*e.g.* three cycles were demonstrated with no detectable irreversible nanoparticle binding).

Significantly, while all of the surface-bound nanoparticle assemblies dissociated

above the T_m of the linking oligonucleotides, the sharpness of these transitions depended on the size of the supported aggregate, Figure 39D-F. Surprisingly, the dissociation of the first nanoparticle layer from the substrate exhibited a transition (Figure 39D, FWHM of the first derivative = 5 °C) that was sharper than that of the same oligonucleotides without nanoparticles in solution, Figure 39C. As more nanoparticle layers were hybridized to the substrate, the melting transition of the oligonucleotide-linked nanoparticles became successively sharper (Figure 39E-F, FWHM of the first derivative = 3 °C), until it matched that of the large nanoparticle network assemblies found in solution. (Gittins et al., *Adv. Mater.* 11:737 (1999); Brust et al., *Langmuir* 14:5425 (1998)). These experiments confirm that more than two nanoparticles and multiple DNA interconnects are required to obtain the optimally sharp melting curves. They also show that the optical changes in this system are completely decoupled from the melting properties (*i.e.*, small aggregates can give sharp transitions but still not change color).

Example 21: Electrical Properties of Gold Nanoparticle Assemblies

Electron transport through DNA has been one of the most intensely debated subjects in chemistry over the past five years. (Kelley et al., *Science* 283:375-381 (1999); Turro et al., *JBIC* 3:201-209 (1998); Lewis et al., *JBIC* 3:215-221 (1998); Ratner, M. *Nature* 397:480-481 (1999); Okahata et al., *J. Am. Chem. Soc.* 120:6165-6166 (1998)) Some claim that DNA is able to efficiently transport electrons, while others believe it to be an insulator.

In a seemingly disparate field of study, a great deal of effort has been devoted to examining the electrical properties of nanoparticle-based materials. (Terrill et al., *J. Am. Chem. Soc.* 117:12537-12548 (1995); Brust et al., *Adv. Mater.* 7:795-797 (1995); Bethell et al., *J. Electroanal. Chem.* 409:137-143 (1996); Musick et al., *Chem. Mater.* 9:1499-1501 (1997); Brust et al., *Langmuir* 14:5425-5429 (1998); Collier et al., *Science* 277:1978-1981 (1997)). Indeed, many groups have explored ways to assemble nanoparticles into two- and three-dimensional networks and have investigated the

electronic properties of such structures. However, virtually nothing is known about the electrical properties of nanoparticle-based materials linked with DNA.

For the first time, in this study, the electrical properties of gold nanoparticle assemblies, formed with different length DNA interconnects have been examined. As shown below, these hybrid inorganic assemblies behave as semiconductors, regardless of oligonucleotide particle interconnect length over a 24 to 72 nucleotide range. The results reported herein indicate that DNA interconnects can be used as chemically specific scaffolding materials for metallic nanoparticles without forming insulating barriers between them and thereby destroying their electrical properties. These results point towards new ways such hybrid assemblies can be exploited as electronic materials.

At the heart of this issue is the following question: Can nanoparticles assembled by DNA still conduct electricity or will the DNA interconnects, which are heavily loaded on each particle, (Mucic, R. C. *Synthetically Programmable Nanoparticle Assembly Using DNA*. Thesis Ph. D., Northwestern University (1999)) act as insulating shells? The conductivities of these materials as a function of temperature, oligonucleotide length, and relative humidity were examined. The DNA-linked nanoparticle structures were characterized by field emission scanning electron microscopy (FE-SEM), synchrotron small angle x-ray scattering (SAXS) experiments, thermal denaturation profiles, and UV-vis spectroscopy.

In a typical experiment (see Figure 40), citrate-stabilized 13 nm gold nanoparticles were modified with 3' and 5' alkanethiol-capped 12-mer oligonucleotides 1 (3' SH $(CH_2)_3O(PO^{2-})$O-ATGCTCAACTCT 5' [SEQ ID NO:59]) and 2 (5' SH $(CH_2)_6O(PO^{2-})$O-CGCATTCAGGAT 3' [SEQ ID NO:50]) as described in Examples 1 and 3. DNA strands with lengths of 24, 48, or 72 bases (3 (5'TACGAGTTGAGAATCCTGAATGCG3' [SEQ ID NO:60]), 4 (5'TACGAGTTGAGACCGTTAAGACGAGGCAATC-ATGCAATCCTGAATGCG 3'[SEQ ID NO:61]), and 5 (5'TACGAGTTGAGACCGTTAAGACGAGGCAATCATGCATATATTGGACGCTTT

ACGGACAACATCCTGAATGCG3'[SEQ ID NO:62]) were used as linkers. Fillers 6 (3'GGCAATTCTGCTCCGTTAGTACGT5'[SEQ ID NO:63]) and 7 (3'GGCAATTCTGCTCCGTTAGTACGTATATAACCTGCGAAATGCCTGTTG5' [SEQ ID NO:64]) were used with the 48 and 72 base linkers. The DNA-modified nanoparticles and DNA linkers and fillers were stored in 0.3 M NaCl, 10 mM phosphate (pH 7) buffer (referred as to 0.3 M PBS) prior to use. To construct nanoparticle assemblies, 1-modified gold nanoparticles (652 µl, 9.7 nM) and 2-modified gold nanoparticles (652 µl, 9.7 nM) were added to linker DNA 3, 4, or 5 (30 µl, 10 nM). After full precipitation, the aggregates were washed with 0.3 M CH_3COONH_4 solution to remove excess linker DNA and NaCl.

Lyophilization (10^{-3} ~10^{-2} torr) of the aggregate to dryness results in pellets and removal of the volatile salt, CH_3COONH_4. Unfunctionalized, citrate-stabilized particles, prepared by the Frens method, (Frens, *Nature Phys. Sci.* 241:20-22 (1973)) were dried as a film and used for comparison purposes. The resulting dried aggregates had a color resembling tarnished brass and were very brittle. FE-SEM images demonstrated that oligonucleotide-modified nanoparticles remained intact upon drying, while citrate-stabilized nanoparticles fused to one another. Significantly, the dried DNA-linked aggregates could be redispersed in 0.3 M PBS buffer (1ml), and exhibited excellent melting properties; heating such a dispersion to 60 °C resulted in dehybridization of the DNA interconnects, yielding a red solution of dispersed nanoparticles. This combined with the FE-SEM data conclusively demonstrated that DNA-modified gold nanoparticles are not irreversibly aggregated upon drying.

The electrical conductivities of the three samples (dried aggregates linked by 3, 4, and 5, respectively) were measured using a computer-controlled, four-probe technique. Electrical contacts consisted of fine gold wires (25 and 60 µm diameter) attached to pellets with gold paste. Samples were cooled in a moderate vacuum (10^{-3} to 10^{-4} torr), and conductivity was measured as the temperature was increased under a dry, low pressure of helium gas. The sample chamber was insulated from light in order to

eliminate possible optoelectronic effects. Excitation currents were kept at or below 100 nA, and the voltage across the entire sample was limited to a maximum of 20 V. Surprisingly, the conductivities of the aggregates formed from all three linkers, ranged from 10^{-5} to 10^{-4} S/cm at room temperature, and they showed similar temperature dependent behavior. The conductivities of the DNA-linked aggregates showed Arrhenius behavior up to about 190°K, which is characteristic of a semiconducting material. This is similar to the behavior of activated electron hopping observed in discontinuous metal island films (Barwinski, *Thin Solid Films* 128:1-9 (1985)). Gold nanoparticle networks linked by alkanedithiols have shown similar temperature dependence (Brust et al., *Adv. Mater.* 7:795-797 (1995); Bethell et al., *J. Electroanal. Chem.* 409:137-143 (1996)). Activation energies of charge transport can be obtained from a plot of ln σ versus 1/T using equation (1).

$$\sigma = \sigma_o \exp[-E_a/(kT)] \qquad (1)$$

The average activation energies calculated from three measurements were 7.4 ± 0.2 meV, 7.5 ± 0.3 meV, and 7.6 ± 0.4 meV for the 24-, 48-, and 72-mer linkers, respectively. Conductivity data from 50°K to 150°K were used for these calculations.

Since the electrical properties of these types of materials should depend on the distance between particles, synchrotron SAXS experiments were used to determine interparticle distances of the dispersed and dried aggregates. The SAXS experiments were performed at the Dupont-Northwestern-Dow Collaborative Access Team (DND-CAT) Sector 5 of the Advanced Photon Source, Argonne National Laboratory. DNA-linked aggregates and dilute samples of DNA-modified colloid were irradiated with an 0.3 micron beam of 1.54 Å radiation, and scattered radiation was collected on a CCD detector. The 2D data were circularly averaged and transformed into a function, $I(s)$, of the scattering vector magnitude, $s = 2\sin(\Theta)/\lambda$, where 2Θ is the scattering angle and λ is the wavelength of the incident radiation. All data were corrected for background

scattering and sample absorption. The first peak position, which is sensitive to interparticle distance, drastically changed from s values of 0.063 nm^{-1}, 0.048nm^{-1}, and 0.037nm^{-1} for the 24-, 48-, and 72-mer linked aggregates, respectively, to an s value of 0.087 nm^{-1} upon drying for all three aggregates structures. This indicates that interparticle distances decreased significantly upon drying, to the point where the particles were almost touching, and that such distances were virtually independent of linker length, while those in solution were highly dependent on linker length. This explains why similar activation energies were observed for the three different linker systems in the dried pellet conductivity experiments. Moreover, it also explains why relatively high conductivities were observed, regardless of how one views the electronic properties of DNA. Unlike the DNA-linked materials, the dried film of citrate-stabilized gold nanoparticles showed metallic behavior. This is consistent with the SEM data, which showed that such particles fuse together.

Above 190°K, the measured conductivities of the DNA-linked samples showed an anomalous dipping behavior. For all samples, the conductivity started to decrease abruptly at approximately 190°K and continued to decrease until approximately 250°K, at which point it increased again. To investigate this unusual behavior in detail, the electrical conductivity was measured as the sample was cooled and warmed repeatedly. Interestingly, the dip in conductivity only occurred in the direction of increasing temperature. Since DNA is hydrophilic and water could potentially affect the electrical properties of the hybrid structures, the effect of relative humidity on the conductivity of the gold aggregates was examined. The resistance increased by a factor of 10 with increasing humidity from 1% to 100%. It should be noted that the characteristic dip was very weak when the sample was kept in vacuum (10^{-6} Torr) for 48 hours prior to the conductivity measurement. From these observations, it was concluded that the unusual dip and subsequent rise in conductivity above 190°K is associated with water melting and the hygroscopic nature of the DNA, which temporarily increased the interparticle distance (until evaporation took place). Consistent with this hypothesis, SAXS

measurements on a dried aggregate that was wetted with 0.3 M PBS buffer showed a 200% increase in interparticle distance (~2 nm).

These studies are important for the following reasons. First, they show that one can use the molecular recognition properties of DNA to assemble nanoparticle-based materials without passivating them or destroying their discrete structural or electrical properties. If these DNA-functionalized particles are to be used to study electrical transport in three-dimensional macroscopic assemblies or even lithographically patterned structures (Piner et al., *Science* 283:661-663 (1999)), it is imperative that their electrical transport properties be delineated. Second, it shows that over a fairly long linker distance (8 ~ 24 nm), the conductivities of the dried assemblies are virtually independent of DNA linker length. This is likely a result of the removal of water and the use of a volatile salt in these experiments; indeed, the free volume created by removal of solvent and salt allows the DNA to be compressed on the surface and close approach of the particles within the aggregates. Third, the aggregates with the DNA-protected nanoparticles behave as semiconductors, while films formed from citrate-stabilized particles exhibit irreversible particle fusion and metallic behavior. Finally, these results point toward the use of these materials in DNA diagnostic applications where sequence specific binding events between nanoparticles functionalized with oligonucleotides and target DNA effect the closing of a circuit and a dramatic increase in conductivity (*i.e.* from an insulator to a semiconductor) (see next example).

Example 22: Detection Of Nucleic Acid Using Gold Electrodes

A method of detecting nucleic acid using gold electrodes is illustrated diagramatically in Figure 41. A glass surface between two gold electrodes was modified with 12-mer oligonucleotides 1 (3' $NH_2(CH_2)_2O(PO^2)$O-ATG-CTC-AAC-TCT [SEQ ID NO:59]) complementary to target DNA 3 (5' TAC GAG TTG AGA ATC CTG AAT GCG [SEQ ID NO:60]) by the method of Guo *et al.*, *Nucleic Acids Res.*, 22, 5456-5465 (1994). Oligonucleotides 2 (5' $SH(CH_2)_6O(PO^2)$O-CGC-ATT-CAG-GAT [SEQ ID NO:50]) were

prepared and attached to 13 nm gold nanoparticles as described in Examples 1 and18 to yield nanoparticles a. Target DNA 3 and nanoparticles a were added to the device. The color of the glass surface turned pink, indicating that target DNA-gold nanoparticle assemblies were formed on the glass substrate. Next, the device was immersed in 0.3 M NaCl, 10 mM phosphate buffer and heated at 40 °C for 1 hour to remove nonspecifically bound DNA, and then treated with a silver staining solution as described in Example 19 for 5 minutes. The resistance of the electrode was 67 kΩ.

For comparison, a control device modified by attaching oligonucleotides 4, instead of oligonucleotides 1, between the electrodes. Oligonucleotides 4 have the same sequence (5' $NH_2(CH_2)_6O(PO^2)$O-CGC-ATT-CAG-GAT [SEQ ID NO:50]) as oligonucleotides 2 on the nanoparticles and will bind to target DNA 3 so as to prevent binding of the nanoparticles. The test was otherwise performed as described above. The resistance was higher than 40 MΩ, the detection limit of the multimeter that was used.

This experiment shows that only complementary target DNA strands form nanoparticle assemblies between the two electrodes of the device, and that the circuit can be completed by nanoparticle hybridization and subsequent silver staining. Therefore, complementary DNA and noncomplementary DNA can be differentiated by measuring conductivity. This format is extendable to substrate arrays (chips) with thousands of pairs of electrodes capable of testing for thousands of different nucleic acids simultaneously.

Example 23: Preparation of Oligonucleotide-Modified Gold Nanoparticles using cyclic disulfide linkers

In this Example, we describe a new cyclic disulfide linker for binding oligonucleotides to gold surfaces, based on steroid disulfide 1a (Figure 42) that is simple to prepare, is broadly useful, and affords gold-oligonucleotide conjugates exhibiting greater stability toward DTT than those prepared using mercaptohexyl linkers. A cyclic disulfide was selected as the reactive site of the anchor unit since ester derivatives of 1,2-

dithiane-4,5-diol were known to form monolayers on gold surfaces (Nuzzo, et al., J. Am. Chem. Soc. 105,4481-4483) and a cyclic disulfide would plausibly bind to the surface through both sulfur atoms (Ulman, A., MRS Bulletin, June 46-51) to give a chelate structure that could exhibit enhanced stability. Epiandrosterone was selected as a linking element since it is a readily available, easily derivatized ketoalcohol and, as a substituent with a large hydrophobic surface, might be expected to help screen the approach of water soluble molecules to the gold surface (Letsinger, et al., J. Am. Chem. Soc. 115, 7535-7536 · Bioconjugate Chem. 9, 826-830).

The oligonucleotide-gold probes used in previous studies were prepared by the reaction of oligonucleotides being terminal mercaptohexyl groups with gold nanoparticles in an aqueous buffer. They proved to be surprisingly robust, functioning well even after heating to 100°C or after storing for 3 years at 5 °C. We have found, however, that these conjugates lose activity as hybridization probes when soaked in solutions containing thiols, which act by displacing the derivatized oligonucleotides from the gold surface. This feature poses a problem when the nanoparticle probes are to be used in a solution containing a thiol, as for example, a PCR solution that contains dithiothreitol (DTT) as a stabilizer for the polymerase enzyme.

(a) General

NMR spectra were recorded on 500 MHz (^{1}H) and 400 MHz (^{31}P; acquisition at 161.9 MHz) Varian spectrometers using $CDCl_3$ as a solvent and TMS as an internal (^{1}H) and H_3PO_4 as an external (^{31}P) standard; chemical shifts are expressed in δ units. MS data were obtained on a Quattro II triple quadrupole mass spectrometer. Automated oligonucleotide synthesis was carried out on a milligene Expedite DNA synthesizer. The analysis for S was made by Oneida Research Services.

(b) Preparation of Steroid-Disulfide Ketal (1a)

The synthetic scheme is shown in Figure 43. A solution of epiandrosterone (0.5g), 1,2-dithiane-4,5-diol (0.28 g), and p-toluenesulfonic acid (15 mg) in toluene (30 mL) was refluxed for 7 h under conditions for removal of water (Dean Stark apparatus);

then the toluene was removed under reduced pressure and the reside taken up in ethyl acetate. This solution was washed with water, dried over sodium sulfate, and concentrated to a syrupy reside, which on standing overnight in pentane/ether afforded compound 1a as a white solid (400 mg); Rf (TLC, silica plate, ether as eluent) 0.5; for comparison, Rf values for epiandrosterone and 1,2-dithiane-4,5-diol obtained under the same conditions are 0.4, and 0.3, respectively. Recrystallization from pentane/ether afforded a white powder, mp 110-112 °C; ^{1}H NMR, δ 3.6 (1H, C^3OH), 3.54-3.39 (2H, m 2OCH of the dithiane ring), 3.2-3.0 (4H, m $2CH_2S$), 2.1-0.7 (29H, m steroid H); mass spectrum (FS^+) calcd for $C_{23}H_{36}O_3S_2$ (M+H) 425.2179, found 425.2151. Anal. ($C_{23}H_{37}O_3S_2$)S: calcd, 15.12; found, 15.26.

(c) Preparation of Steroid-Disulfide Ketal Phosphoramidite Derivative (1b)

Compound 1a (100 mg) was dissolved in THF (3 mL) and cooled in a dry ice alcohol bath. N,N-diisopropylethylamine (80 μL) and β- cyanoethyl chlorodiisopropylphosphoramidite (80 μL) were added successively; then the mixture was warmed to room temperature, stirred for 2 h, mixed with ethyl acetate (100 mL), washed with 5% aq. $NaHCO_3$ and with water, dried over sodium sulfate, and concentrated to dryness. The residue was taken up in the minimum amount of dichloromethane, precipitated at –70 °C by addition of hexane, and dried under vacuum; yield 100 mg; ^{31}P NMR 146.02.

(d) Preparation of 5'-Modified Oligonucleotides and Nanoparticle Conjugates

5'-Modified oligonucleotides Ic1 and Ic2 were constructed on CPG supports using conventional phosphoramidite chemistry, except that compound 1b (Figure 42) was employed in the final phosphitilation step. Products were cleaved from the support by treatment with concentrated NH_4OH for 16 h at 55 °C. The oligonucleotides were purified by reversed phase HPLC on a Dionex DX500 system equipped with a Hewlett Packard ODS Hypersil column (4.6 x 200 nm, 5 μm particle size) using TEAA buffer (pH 7.0) and a 1%/min gradient of 95% CH_3CN/5% 0.03 TEAA at a flow rate of 1

mL/min. A nice feature of the hydrophobic steroid group is that the capped derivatives separate cleanly from uncapped oligomers. Sulfur derivatized oligonucleotides IIa, IIc1 and IIc2 (Figure 43) were prepared similarly using the commercially available "5'-Thiol-Modifier reagent" (C6 Glen Research) and cleavage of the trityl protecting group with silver nitrate as previously described (Storhoff, J.J., et al., J. Am. Chem. Soc. 120, 1959-1964). For preparation of disulfide IIIc1, "Thiol-Modifier C6 S-S" (Glen Research) was used in the final phosphitilation and the terminal dimethoxytrityl group was cleaved with aqueous 80% acetic acid.

Each of the modified oligonucleotides was immobilized on ~ 13 nm gold nanoparticles by the procedure used for anchoring oligonucleotides through a mercaptohexyl head group (Storhoff et al. (1998) J. Am. Chem. Soc. 120, 1959-1964). This involved soaking citrate stabilized nanoparticles (~ 13 nm in diameter) for 56 hours in a buffer-salt solution containing an oligonucleotide bearing a terminal sulfur substituent (HS- or acyclic or cyclic disulfide) followed by addition of NaCL to 0.1 M and 24h of standing. The nanoparticles were pelleted by centrifugation, the supernatant solution was removed, and the nanoparticles were washed, resuspended in buffer, recentrifuged, and then suspended in 0.1 M NaCl, 10 mM phosphate. This procedure afforded the nanoparticle oligonucleotide conjugates free from the excess sulfurized oligonucleotides employed in the loading process.

(e) Hybridization

To evaluate the utility of nanoparticle-oligonucleotide probes containing the disulfide-steroid anchor we prepared probes Ic1, Ic2, IIc1, IIc2, and IIIc1 by immobilizing the modified oligonucleotides on gold nanoparticles (Figure 2). The oligomers in a given series have the same nucleotide sequence but differ in structure of the 5'-head group Y. Conjugates Ic1 and Ic2 have steroid-disulfide head groups; IIc1, IIc2, mercaptohexyl head groups; and IIIc1, acylic disulfide head groups. The $(dA)_{20}$ chains serve as spacers between the gold and the oligonucleotide recognition regions to facilitate hybridization. Many of the sulfur derivatized oligomers bind to each

nanoparticle. Hybridization of pairs of nanoparticle probes with target oligonucleotides leads to formation of three dimensional networks and a change in color from red to blue-gray (Mucic, R. C., et al., J. Am. Chem. Soc. 120, 12674-12675).

Hybridization of the probes was examined using a 79-mer oligonucleotide targets, containing sequences complementary to the probes (Figure 43). The reactions were carried out at room temperature by adding 1 μL of the target solution (10 pmol of IV) to colloidal solutions of the probe pairs Ic1, Ic2, and IIc1 and IIc2, and IIIc1 and IIc2 (50 μL and 1.0 A_{520} Unit of each nanoparticle probe) in 0.5 M NaCl, 10 mM phosphate (pH 7.0). At times 10 seconds, 5 minutes, and 10 minutes, aliquots (3 μL) were removed and spotted on a C-18 reversed phase TLC plate. The various probe pairs all behaved the same: the spots for the 10 second reactions were red, indicative of free nanoparticles; those for the 10 minute reactions were deep blue-gray, characteristic of aggregates of nanoparticles; and the 5 minute reactions afforded spots with a reddish blue color, indicative of a mixture of non-associated and associated nanoparticles. In agreement with previous observations for aggregation of nanoparticles effected by hybridization of oligonucleotides (Storhoff, J. J., et al., J. Am. Chem. Soc. 120, 1959-1964; Elghanian, et al., Science, 277, 1078-1081; Mucic, R. C., et al., J. Am. Chem. Soc. 120, 12674-12675; Mitchell, G. P., J. Am. Chem. Soc. 121, 8122-8123), the reactions were reversible. Thus, warming the aggregate mixture to 90 °C (above the dissociation temperature for the oligomers linking the nanoparticles together) and spotting while hot afforded a red spot. For control experiments in which the oligonucleotide target was omitted or was not complementary to the probes, the color was red under all conditions.

We conclude that the nanoparticle conjugates generated via the steroid-disulfide anchor function effectively as hybridization probes. Moreover, as judged by the spot test, the conjugates with the various anchor units react with the target oligonucleotides at comparable rates. This feature is consistent with expectations for probes having comparable densities of oligonucleotides on the surface of the nanoparticles and nucleotide recognition regions relatively far removed from the 5'-head groups.

(f) Reaction of Nanoparticle Probes with Dithiothreitol.

Addition of thiols to colloidal solutions of gold nanoparticles or gold nanoparticles loaded with mercaptohexyl-oligonucleotides leads to aggregation of the nanoparticles. The color changes from red to deep blue, and on standing a dark precipitate settles out. As demonstrated with experiments with nanoparticles bearing fluorescently labeled oligonucleotides, the thiol displaces the mercaptoalkyl-oligonucleotides bound at the gold surface (Mucic, R. C., (1999) Synthetic Programmable Nanoparticle Assembly Using DNA, PhD Thesis, Northwestern University). In contrast to aggregation induced by hybridization of oligonucleotide-nanoparticle conjugates, these reactions are irreversible; neither heating nor addition of NaOH disassembles the aggregates.

We have used this color to monitor the reaction of DTT with probes prepared with the steroid cyclic disulfide, the mercaptohexyl, and the acyclic disulfide head groups. The experiments were carried out by adding 1 μL of 1M DTT in water to 100 μL of the nanoparticle-oligonucleotide probe solution (2 A_{520} Units of nanoparticles) in 0.5 M NaCl and 10 mM phosphate (pH 7.0), then spotting 3 μL aliquots on a TLC plate at various times and observing the color. As shown in Table 1, colloidal probes derived from oligonucleotides with the mercaptohexyl **(IIc1 and IIc2)** and acyclic oligonucleotide headgroups (IIIc1) reacted rapidly. A red-blue spot was obtained in 20 seconds and a strong blue spot within 5 minutes. By 100 minutes, most of the gold had precipitated. In contrast, no color change was observed for the reaction of the probes prepared with the steroid-cyclic disulfide head group (Ic1, Ic2) within 40 minutes. It took 100 minutes to reach the same color obtained with probes prepared with IIc1, IIc2, or with IIIc1 in 20 seconds. On this basis, we estimate that the rate of reaction of the steroid disulfide probes with DTT is of the order of $1/300^{th}$ that of the other probes. The probe prepared from the acyclic disulfide anchor 3c reacts at about the same rate as the probes prepared with the mercaptohexyl anchor. The latter result is not surprising in view of evidence that the reaction of an acyclic disulfide with gold probably involves cleavage of the S-S

bond (Zhong, C. J., Langmuir, 15, 518-525). Accordingly, an oligonucleotide with an acyclic head group would likely be linked to gold through a single sulfur atom, as in the case of mercaptohexyl-oligonucleotide derivatives.

To see if probes prepared from 1c1 and 1c2 in fact still serve as hybridization probes after standing in the presence of DTT, we treated two samples of mixtures of the probes with DTT under the conditions used for the reactions in Table I. After 30 minutes, 1 μL of a solution of the 79-mer target oligonucleotide (10 pmol) was added to one. Both samples were frozen quickly, allowed to thaw, and assayed by the spot test. The spot for the sample containing the target was blue and that for the control lacking the target was red, demonstrating that these nanoparticle conjugates were not only stable but also effective as probes after exposure to DTT under conditions causing aggregation of probes derived from the mercaptohexyl or linear disulfide anchoring group.

Table 1. Colors from reactions of Gold Nanoparticle Probes with DTT

1c1 + 1c2	Time 0	20 sec	5 min	40 min	100 min
	red	red	red	red	red-blue
IIc1 + IIc2	red	red-blue	blue	blue	(black prec.)
IIIc1	red	red-blue	blue	blue	(black prec.)

(g) Conclusion

Gold nanoparticle-oligonucleotide conjugates made using this cyclic disulfide linker serve as effective probes for detecting specific oligonucleotide sequences, and they exhibit much greater stability toward dithiothreitol than corresponding conjugates prepared with the conventional mercaptohexyl group or an acyclic disulfide unit. The high stability toward thiol deactivation likely results, in part at least, from anchoring each

oligonucleotide to gold through two sulfur atoms.

Example 24: Preparation of Oligonucleotide-Modified Gold Nanoparticles using a simple cyclic disulfide linker.

In this Example, we prepared a non-steroid cyclic disulfide linker and oligonucleotide-nanoparticle probes from this linker and evaluated the probes stability in the presence of thiol-containing solutions relative to probes prepared with steroidal cyclic disulfide and alkyl thiol linkers. Procedures have been described for preparing probes for detecting DNA or RNA sequences by binding oligonucleotides to gold nanoparticles using alkylthiol anchor groups, I, Figure 44 [C. A. Mirkin et al., Nature, 382, 607 (1996); Storhoff, et al. J. Am. Chem. Soc., 120, 1959 (1998)] or a steroid cyclic disulfide anchor group, II, Figure 44 [R. L. Letsinger et al., Bioconjugate Chemistry, 11, 289 (2000)]. As probes, the conjugates prepared using the steroid cyclic disulfide linker have proved advantageous in that they are much more stable in the presence of thiol compounds, such as mercaptoethanol or dithiothreitol (DTT), than are conjugates prepared using an alkylthiol anchor. This feature is important since PCR solutions employed in amplifying DNA samples for detection contain small amounts of DTT to protect the enzyme. For simple and rapid detection of PCR products it is desirable to use probes with high stability toward DTT so that the test can be carried out directly in the PCR solution without having to first isolate the amplified DNA.

Two features distinguish the steroid cyclic anchor (compound I, Figure 42): (1) the cyclic disulfide, which can in principle provide two binding sites that could act cooperatively in holding a given oligonucleotide at the gold surface, and (2) the steroid unit which could stabilize neighboring chains on the gold by hydrophobic interactions (see for example, R. L. Letsinger et al., J. Am. Chem. Soc. 115, 7535 (1993). To assess the importance of these contributions we have prepared and examined gold conjugates anchored by a cyclic disulfide lacking a steroid group, compound IIIc (Figure 44).

Compound 2a, prepared by heating trans-1,2-dithiane-4,5-dithiol with acetol in

toluene, was converted to a cyanoethyl N,N-di-i-propyl phosphoramidite reagent, 2h , which was employed in the final coupling step in the synthesis of modified oligonucleotides 2c1 and 2c2. One gold conjugate probe was prepared by treating a gold colloid solution with 2c1 and an equimolar amount of 2d, which serves a diluent on the gold surface. A companion probe was made from 2c2 and 2d in the same way. These nanoparticle conjugates were stable in a range of solutions of sodium chloride (0.1, 0.3, 0.5, 0.7 M), both on standing and on freezing and thawing.

(a) Preparation of compounds 2a, 2b, 2c1 and 2c2.

Compounds 2a was prepared as described in Example 23. Phosphitilation of 2a and synthesis of of oligonucleotides 2c1 and 2c2 were carried out as described previously for the steroid cyclic disulfide derivatives in Example 23 and elsewhere [R. L. Letsinger et al., Bioconjugate Chemistry, 11, 289 (2000), the disclosure which is incorporated by reference in its entirety]. The time of reaction in the step involving condensation of IIIb with the oligomer on the CPG support was 10 min.

(b) Preparation of Gold-Oligonucleotide Conjugates.

Equimolar amount of oligonucleotides 2c1 and 2d or 2c2 and 2d were added to 13 nm gold colloids (~ 10 nM) to provide solutions containing 1.7 μmole/mL of each oligonucleotide. The solutions were stored in the dark for 24 h; then salts were added to make the solutions 0.3 M in NaCl, 10 mM in phosphate (pH 7.0), and 0.01% in sodium azide. After 24 h the NaCl concentration was increased to 0.8 M and the solution were allowed to stand for another 24 h. The colloid was then filtered to remove any aggregates and the solution was centrifuged to collect the nanoparticles. The pellets were washed with nanopure water, recentrifuged and redispersed in 0.1 M NaCl, 10 mM phosphate buffer (pH 7.0), 0.01 % sodium azide.

(c) Reaction of nanoparticles probes with dithiothreitol

Displacement studies were carried out at room temperature (22°C) by adding 2 μL of 0.1 M DTT to 20 μL of a mixture of equal volumes of the colloidal conjugates obtained from 2c1 and 2c2. Aliquots (3 μL) were periodically removed and spotted onto

a white Nylon membrane. Initially the spots were red. Displacement of the oligonucleotide sulfur derivatives from the gold by DTT led to mixtures that afforded a blue-gray spot in the spot test. The time for displacement by DTT was taken as the time for the mixture to give a strong blue-gray color in a spot test. For the mixture of conjugates derived from 2c1 and 2c2 this time was 10 hours.

For comparison, oligonucleotide conjugates were similarly prepared from oligonucleotide sequences c1 and c2 (Figure 44) using the mercaptohexyl anchor (compound 2, Figure 42) and the steroid cyclic disulfide anchor (compound 1, Figure 42). The reaction times for the conjugates prepared from the monothiol derivative (2, Figure 42) and the steroid cyclic disulfide as measured by the time to afford a blue-gray spot, were 5 minutes and 53 hours, respectively. These values correspond to relative stabilities of 1, ~60, and ~300 for the nanoparticle oligonucleotide conjugates derived from the mercaptoalkyl derivatives, the non-steroid cyclic disulfide, and the steroid cyclic disulfide derivatives, respectively. The results show that the cyclic disulfide anchor unit itself is sufficient to afford high stability relative to the mercaptoalkyl group in these systems. The large hydrophobic group in nanoparticle conjugates derived from compound 1 also appears to play a role in enhancing stability toward thiol displacement of the oligonucleotides.

Example 25: Preparation of Oligonucleotide-Modified Gold Nanoparticles

In this Example, we evaluated the stability of a new acyclic disulfide linker, a tri thiol, relative to alkyl thiol and steroidal cyclic disulfide linkers in the presence of thiol-containing solutions. For comparison, oligonucleotides with a mercaptohexyl anchor (compound 5, Figure 45) and with a steroid cyclic disulfide anchor (compound 6, Figure 45) were prepared.

(a) Synthesis and characterization of 5'-tri-mercaptoalkyl oligonucleotide.

The 5'-trimercaptoalkythiol oligonucleotide was synthesized as shown in Figure 47. Trembler phosphoramidite 7 (Glen Research Inc, Sterling, VA), Figure 45, and Thiol-

modifier C6 S-S phosphoramidite, were sequentially coupled to the 5' end of a protected oligomer still bound to the CPG support. The product was cleaved from the CPG and purified as describe above. The retention time for tri-thiol oligonucleotide with three DMT group on the end is approximately 64 minutes. 5'-DMT groups were subsequently removed by dissolving the oligonucleotide in 80% acetic acid for 30 min. followed by evaporation. The oligonucleotide was redissolved in 500 µL nanopure water and the solution was extracted with ethyl acetate (3 x 300 µL). After evaporation of the solvent, the oligonucleotide was obtained as a white solid. The retention time of this 5'- tri-disulfide oligonucleotide with no DMT group was around 35 minutes on reverse phase columns while 24 minutes on the ion-exchange column. These peaks were both more than 97% of the area in the spectra, which indicates the high purity of the oligonucleotide. The formula weight of oligonucleotide 8 (Figure 45) was obtained by electrospray MS (calculated: 12242.85, found 12244.1). The three disulfide groups on the DNA stand were reduced tri thiol groups as described above for 5' monothiol DNA; then the oligonucleotide was purified though a NAP-5 column.

(b) Preparation of 5'-thiol or disulfide DNA modified gold nanoparticle

Gold nanoparticles were used as purchased from Vector Laboratories (Burlingame, CA). To 10 mL of 30nm gold colloid was added 5 OD of thiol modified DNA. The solution was brought to 0.3 M NaCl/10 mM sodium phosphate buffer (pH 7) (PBS) gradually. Then the nanoparticles were thrown down by centrifugation. After removing the colorless supernatant, the red oily precipitate was redispersed in 10 mL of fresh PBS buffer. The colloid was washed twice more using 10ml fresh PBS buffer by repeating this process.

(c) Stability test of thiol-DNA modified gold nanoparticles

Solid DTT was added to 600 uL solutions of the different types of thiol- or disulfide DNA modified 30 nm gold nanoparticle colloids until the DTT concentration was 0.017M. As DTT displaces the oligonucleotides, the color of the colloid turns from red to blue.UV/VIS spectra were taken as a function of time. The absorbance at ~528nm

associated with dispersed 30nm gold particles began to decrease and a broad band at 700nm began to grow. The band at 700 nm is associated with colloid aggregation. As shown in Figure 48, single thiol oligonucleotide (1)-modified 30 nm gold particles quickly form an aggregate in 0.017M DTT; after 1.5 hours, the colloid totally turns blue. The solution containing disulfide oligonucleotide (4)-modified nanoparticles turns blue after 20 hours under identical conditions. For the trithiol-oligonucleotide (cleaved 6) modified nanoparticles, it took 40 h to turn the solution blue.

All patents, patent applications and references cited herein are hereby incorporated by reference in their entirety.

WE CLAIM:

1. A method of detecting a nucleic acid having at least two portions comprising:

providing a type of nanoparticles having oligonucleotides attached thereto, the oligonucleotides on each nanoparticle having a sequence complementary to the sequence of at least two portions of the nucleic acid;

contacting the nucleic acid and the nanoparticles under conditions effective to allow hybridization of the oligonucleotides on the nanoparticles with the two or more portions of the nucleic acid; and

observing a detectable change brought about by hybridization of the oligonucleotides on the nanoparticles with the nucleic acid.

2. A method of detecting nucleic acid having at least two portions comprising:

contacting the nucleic acid with at least two types of nanoparticles having oligonucleotides attached thereto, the oligonucleotides on the first type of nanoparticles having a sequence complementary to a first portion of the sequence of the nucleic acid, the oligonucleotides on the second type of nanoparticles having a sequence complementary to a second portion of the sequence of the nucleic acid, the contacting taking place under conditions effective to allow hybridization of the oligonucleotides on the nanoparticles with the nucleic acid; and

observing a detectable change brought about by hybridization of the oligonucleotides on the nanoparticles with the nucleic acid.

3. The method of Claim 2 wherein the contacting conditions include freezing and thawing.

4. The method of Claim 2 wherein the contacting conditions include heating.

5. The method of Claim 2 wherein the detectable change is observed on a solid surface.

6. The method of Claim 2 wherein the detectable change is a color change observable with the naked eye.

7. The method of Claim 6 wherein the color change is observed on a solid surface.

8. The method of Claim 2 wherein the nanoparticles are made of gold.

9. The method of Claim 2 wherein the oligonucleotides attached to the nanoparticles are labeled on their ends not attached to the nanoparticles with molecules that produce a detectable change upon hybridization of the oligonucleotides on the nanoparticles with the nucleic acid.

10. The method of Claim 9 wherein the nanoparticles are metallic or semiconductor nanoparticles and the oligonucleotides attached to the nanoparticles are labeled with fluorescent molecules.

11. The method of Claim 2 wherein:

the nucleic acid has a third portion located between the first and second portions, and the sequences of the oligonucleotides on the nanoparticles do not include sequences complementary to this third portion of the nucleic acid; and

the nucleic acid is further contacted with a filler oligonucleotide having a sequence complementary to this third portion of the nucleic acid, the contacting taking place under conditions effective to allow hybridization of the filler oligonucleotide with

the nucleic acid.

12. The method of Claim 2 wherein the nucleic acid is viral RNA or DNA.

13. The method of Claim 2 wherein the nucleic acid is a gene associated with a disease.

14. The method of Claim 2 wherein the nucleic acid is a bacterial DNA.

15. The method of Claim 2 wherein the nucleic acid is a fungal DNA.

16. The method of Claim 2 wherein the nucleic acid is a synthetic DNA, a synthetic RNA, a structurally-modified natural or synthetic RNA, or a structurally-modified natural or synthetic DNA.

17. The method of Claim 2 wherein the nucleic acid is from a biological source.

18. The method of Claim 2 wherein the nucleic acid is a product of a polymerase chain reaction amplification.

19. The method of Claim 2 wherein the nucleic acid is contacted with the first and second types of nanoparticles simultaneously.

20. The method of Claim 2 wherein the nucleic acid is contacted and hybridized with the oligonucleotides on the first type of nanoparticles before being contacted with the second type of nanoparticles.

21. The method of Claim 20 wherein the first type of nanoparticles is attached

to a substrate.

22. The method of Claim 2 wherein the nucleic acid is double-stranded and hybridization with the oligonucleotides on the nanoparticles results in the production of a triple-stranded complex.

23. A method of detecting nucleic acid having at least two portions comprising:

providing a substrate having a first type of nanoparticles attached thereto, the nanoparticles having oligonucleotides attached thereto, the oligonucleotides having a sequence complementary to a first portion of the sequence of a nucleic acid to be detected;

contacting said nucleic acid with the nanoparticles attached to the substrate under conditions effective to allow hybridization of the oligonucleotides on the nanoparticles with said nucleic acid;

providing a second type of nanoparticles having oligonucleotides attached thereto, the oligonucleotides having a sequence complementary to one or more other portions of the sequence of said nucleic acid;

contacting said nucleic acid bound to the substrate with the second type of nanoparticles under conditions effective to allow hybridization of the oligonucleotides on the second type of nanoparticles with said nucleic acid; and

observing a detectable change.

24. The method of Claim 23 wherein the substrate has a plurality of types of nanoparticles attached to it in an array to allow for the detection of multiple portions of a single nucleic acid, the detection of multiple different nucleic acids, or both.

25. A method of detecting nucleic acid having at least two portions

comprising:

providing a substrate having a first type of nanoparticles attached thereto, the nanoparticles having oligonucleotides attached thereto, the oligonucleotides having a sequence complementary to a first portion of the sequence of a nucleic acid to be detected;

contacting said nucleic acid with the nanoparticles attached to the substrate under conditions effective to allow hybridization of the oligonucleotides on the nanoparticles with said nucleic acid;

providing a second type of nanoparticles having oligonucleotides attached thereto, the oligonucleotides having a sequence complementary to one or more other portions of the sequence of said nucleic acid;

contacting said nucleic acid bound to the substrate with the second type of nanoparticles under conditions effective to allow hybridization of the oligonucleotides on the second type of nanoparticles with said nucleic acid;

providing a binding oligonucleotide having a selected sequence having at least two portions, the first portion being complementary to at least a portion of the sequence of the oligonucleotides on the second type of nanoparticles;

contacting the binding oligonucleotide with the second type of nanoparticles bound to the substrate under conditions effective to allow hybridization of the binding oligonucleotide to the oligonucleotides on the nanoparticles;

providing a third type of nanoparticles having oligonucleotides attached thereto, the oligonucleotides having a sequence complementary to the sequence of a second portion of the binding oligonucleotide;

contacting the third type of nanoparticles with the binding oligonucleotide bound to the substrate under conditions effective to allow hybridization of the binding oligonucleotide to the oligonucleotides on the nanoparticles; and

observing a detectable change.

26. The method of Claim 25 wherein the substrate has a plurality of types of nanoparticles attached to it in an array to allow for the detection of multiple portions of a single nucleic acid, the detection of multiple different nucleic acids, or both.

27. A method of detecting nucleic acid having at least two portions comprising:

contacting a nucleic acid to be detected with a substrate having oligonucleotides attached thereto, the oligonucleotides having a sequence complementary to a first portion of the sequence of said nucleic acid, the contacting taking place under conditions effective to allow hybridization of the oligonucleotides on the substrate with said nucleic acid;

contacting said nucleic acid bound to the substrate with a first type of nanoparticles having one or more types of oligonucleotides attached thereto, at least one of the types of oligonucleotides having a sequence complementary to a second portion of the sequence of said nucleic acid, the contacting taking place under conditions effective to allow hybridization of the oligonucleotides on the nanoparticles with said nucleic acid;

contacting the first type of nanoparticles bound to the substrate with a second type of nanoparticles having oligonucleotides attached thereto, the oligonucleotides on the second type of nanoparticles having a sequence complementary to at least a portion of the sequence of one of the types of oligonucleotides on the first type of nanoparticles, the contacting taking place under conditions effective to allow hybridization of the oligonucleotides on the first and second types of nanoparticles; and

observing a detectable change.

28. The method of Claim 27 wherein the first type of nanoparticles has only one type of oligonucleotides attached thereto, the oligonucleotides having a sequence complementary to the second portion of the sequence of said nucleic acid and to at least a portion of the sequence of the oligonucleotides on the second type of nanoparticles.

29. The method of Claim 28 further comprising contacting the second type of nanoparticles bound to the substrate with the first type of nanoparticles, the contacting taking place under conditions effective to allow hybridization of the oligonucleotides on the first and second types of nanoparticles.

30. The method of Claim 27 wherein the first type of nanoparticles has at least two types of oligonucleotides attached thereto, the first type of oligonucleotides having a sequence complementary to the second portion of the sequence of said nucleic acid, and the second type of oligonucleotides having a sequence complementary to the sequence of at least a portion of the oligonucleotides on the second type of nanoparticles.

31. The method of Claim 30 further comprising contacting the second type of nanoparticles bound to the substrate with the first type of nanoparticles, the contacting taking place under conditions effective to allow hybridization of the oligonucleotides on the first and second types of nanoparticles.

32. The method of Claim 27 wherein the substrate has a plurality of types of oligonucleotides attached to it in an array to allow for the detection of multiple portions of a single nucleic acid, the detection of multiple different nucleic acids, or both.

33. The method of any one of Claims 23-32 wherein the substrate is a transparent substrate or an opaque white substrate.

34. The method of Claim 33 wherein the detectable change is the formation of dark areas on the substrate.

35. The method of any one of Claims 23-32 wherein the nanoparticles are

made of gold.

36. The method of any one of Claims 23-32 wherein the substrate is contacted with silver stain to produce the detectable change.

37. The method of any one of Claims 23-32 wherein the detectable change is observed with an optical scanner.

38. A method of detecting nucleic acid having at least two portions comprising:

contacting a nucleic acid to be detected with a substrate having oligonucleotides attached thereto, the oligonucleotides having a sequence complementary to a first portion of the sequence of said nucleic acid, the contacting taking place under conditions effective to allow hybridization of the oligonucleotides on the substrate with said nucleic acid;

contacting said nucleic acid bound to the substrate with a type of nanoparticles having oligonucleotides attached thereto, the oligonucleotides having a sequence complementary to a second portion of the sequence of said nucleic acid, the contacting taking place under conditions effective to allow hybridization of the oligonucleotides on the nanoparticles with said nucleic acid;

contacting the substrate with silver stain to produce a detectable change; and

observing the detectable change.

39. The method of Claim 38 wherein the nanoparticles are made of a noble metal.

40. The method of Claim 39 wherein the nanoparticles are made of gold or silver.

41. The method of Claim 38 wherein the substrate has a plurality of types of oligonucleotides attached to it in an array to allow for the detection of multiple portions of a single nucleic acid, the detection of multiple different nucleic acids, or both.

42. The method of any one of Claims 38-41 wherein the detectable change is observed with an optical scanner.

43. A method of detecting nucleic acid having at least two portions comprising:

contacting a nucleic acid to be detected with a substrate having oligonucleotides attached thereto, the oligonucleotides having a sequence complementary to a first portion of the sequence of said nucleic acid, the contacting taking place under conditions effective to allow hybridization of the oligonucleotides on the substrate with said nucleic acid;

contacting said nucleic acid bound to the substrate with liposomes having oligonucleotides attached thereto, the oligonucleotides having a sequence complementary to a portion of the sequence of said nucleic acid, the contacting taking place under conditions effective to allow hybridization of the oligonucleotides on the liposomes with said nucleic acid;

contacting the liposomes bound to the substrate with a first type of nanoparticles having at least a first type oligonucleotides attached thereto, the first type of oligonucleotides having a hydrophobic group attached to the end not attached to the nanoparticles, the contacting taking place under conditions effective to allow attachment of the oligonucleotides on the nanoparticles to the liposomes as a result of hydrophobic interactions; and

observing a detectable change.

44. A method of detecting nucleic acid having at least two portions

comprising

contacting a nucleic acid to be detected with a substrate having oligonucleotides attached thereto, the oligonucleotides having a sequence complementary to a first portion of the sequence of said nucleic acid, the contacting taking place under conditions effective to allow hybridization of the oligonucleotides on the substrate with said nucleic acid;

contacting said nucleic acid bound to the substrate with liposomes having oligonucleotides attached thereto, the oligonucleotides having a sequence complementary to a portion of the sequence of said nucleic acid, the contacting taking place under conditions effective to allow hybridization of the oligonucleotides on the liposomes with said nucleic acid;

contacting the liposomes bound to the substrate with a first type of nanoparticles having at least a first type oligonucleotides attached thereto, the first type of oligonucleotides having a hydrophobic group attached to the end not attached to the nanoparticles, the contacting taking place under conditions effective to allow attachment of the oligonucleotides on the nanoparticles to the liposomes as a result of hydrophobic interactions;

contacting the first type of nanoparticles bound to the liposomes with a second type of nanoparticles having oligonucleotides attached thereto,

the first type of nanoparticles having a second type of oligonucleotides attached thereto which have a sequence complementary to at least a portion of the sequence of the oligonucleotides on the second type of nanoparticles,

the oligonucleotides on the second type of nanoparticles having a sequence complementary to at least a portion of the sequence of the second type of oligonucleotides on the first type of nanoparticles,

the contacting taking place under conditions effective to allow hybridization of the oligonucleotides on the first and second types of nanoparticles; and

observing a detectable change.

45. The method of Claim 43 or 44 wherein the substrate has a plurality of types of oligonucleotides attached to it in an array to allow for the detection of multiple portions of a single nucleic acid, the detection of multiple different nucleic acids, or both.

46. The method of Claim 43 or 44 wherein the nanoparticles are made of gold.

47. The method of Claim 43 or 44 wherein the substrate is contacted with silver stain to produce the detectable change.

48. The method of any one of Claims 43 or 44 wherein the detectable change is observed with an optical scanner.

49. A method of detecting nucleic acid having at least two portions comprising:

providing a substrate having a first type of nanoparticles attached thereto, the nanoparticles having oligonucleotides attached thereto, the oligonucleotides having a sequence complementary to a first portion of the sequence of a nucleic acid to be detected;

contacting said nucleic acid with the nanoparticles attached to the substrate under conditions effective to allow hybridization of the oligonucleotides on the nanoparticles with said nucleic acid;

providing an aggregate probe comprising at least two types of nanoparticles having oligonucleotides attached thereto, the nanoparticles of the aggregate probe being bound to each other as a result of the hybridization of some of the oligonucleotides attached to them, at least one of the types of nanoparticles of the aggregate probe having oligonucleotides attached thereto which have a sequence complementary to a second portion of the sequence of said nucleic acid;

contacting said nucleic acid bound to the substrate with the aggregate probe under conditions effective to allow hybridization of the oligonucleotides on the aggregate probe with said nucleic acid; and

observing a detectable change.

50. The method of Claim 49 wherein the substrate has a plurality of types of nanoparticles attached to it in an array to allow for the detection of multiple portions of a single nucleic acid, the detection of multiple different nucleic acids, or both.

51. A method of detecting nucleic acid having at least two portions comprising:

providing a substrate having oligonucleotides attached thereto, the oligonucleotides having a sequence complementary to a first portion of the sequence of a nucleic acid to be detected;

providing an aggregate probe comprising at least two types of nanoparticles having oligonucleotides attached thereto, the nanoparticles of the aggregate probe being bound to each other as a result of the hybridization of some of the oligonucleotides attached to them, at least one of the types of nanoparticles of the aggregate probe having oligonucleotides attached thereto which have a sequence complementary to a second portion of the sequence of said nucleic acid;

contacting said nucleic acid, the substrate and the aggregate probe under conditions effective to allow hybridization of said nucleic acid with the oligonucleotides on the aggregate probe and with the oligonucleotides on the substrate; and

observing a detectable change.

52. The method of Claim 51 wherein said nucleic acid is contacted with the substrate so that said nucleic acid hybridizes with the oligonucleotides on the substrate, and said nucleic acid bound to the substrate is then contacted with the aggregate probe so

that said nucleic acid hybridizes with the oligonucleotides on the aggregate probe.

53. The method of Claim 51 wherein said nucleic acid is contacted with the aggregate probe so that said nucleic acid hybridizes with the oligonucleotides on the aggregate probe, and said nucleic acid bound to the aggregate probe is then contacted with the substrate so that said nucleic acid hybridizes with the oligonucleotides on the substrate.

54. The method of Claim 51 wherein said nucleic acid is contacted simultaneously with the aggregate probe and the substrate.

55. The method of Claim 51 wherein the substrate has a plurality of types of oligonucleotides attached to it in an array to allow for the detection of multiple portions of a single nucleic acid, the detection of multiple different nucleic acids, or both.

56. A method of detecting nucleic acid having at least two portions comprising:

providing a substrate having oligonucleotides attached thereto;

providing an aggregate probe comprising at least two types of nanoparticles having oligonucleotides attached thereto, the nanoparticles of the aggregate probe being bound to each other as a result of the hybridization of some of the oligonucleotides attached to them, at least one of the types of nanoparticles of the aggregate probe having oligonucleotides attached thereto which have a sequence complementary to a first portion of the sequence of a nucleic acid to be detected;

providing a type of nanoparticles having at least two types of oligonucleotides attached thereto, the first type of oligonucleotides having a sequence complementary to a second portion of the sequence of said nucleic acid, the second type of oligonucleotides having a sequence complementary to at least a portion of the

sequence of the oligonucleotides attached to the substrate;

contacting said nucleic acid, the aggregate probe, the nanoparticles and the substrate, the contacting taking place under conditions effective to allow hybridization of said nucleic acid with the oligonucleotides on the aggregate probe and on the nanoparticles and hybridization of the oligonucleotides on the nanoparticles with the oligonucleotides on the substrate; and

observing a detectable change.

57. The method of Claim 56 wherein said nucleic acid is contacted with the aggregate probe and the nanoparticles so that said nucleic acid hybridizes with the oligonucleotides on the aggregate probe and with the oligonucleotides on the nanoparticles, and said nucleic acid bound to the aggregate probe and nanoparticles is then contacted with the substrate so that the oligonucleotides on the nanoparticles hybridize with the oligonucleotides on the substrate.

58. The method of Claim 56 wherein said nucleic acid is contacted with the aggregate probe so that said nucleic acid hybridizes with the oligonucleotides on the aggregate probe, said nucleic acid bound to the aggregate probe is then contacted with the nanoparticles so that said nucleic acid hybridizes with the oligonucleotides on the nanoparticles, and said nucleic acid bound to the aggregate probe and nanoparticles is then contacted with the substrate so that the oligonucleotides on the nanoparticles hybridize with the oligonucleotides on the substrate.

59. The method of Claim 56 wherein said nucleic acid is contacted with the aggregate probe so that said nucleic acid hybridizes with the oligonucleotides on the aggregate probe, the nanoparticles are contacted with the substrate so that the oligonucleotides on the nanoparticles hybridize with the oligonucleotides on the substrate, and said nucleic acid bound to the aggregate probe is then contacted with the

nanoparticles bound to the substrate so that said nucleic acid hybridizes with the oligonucleotides on the nanoparticles.

60. The method of Claim 56 wherein the substrate has the oligonucleotides attached to it in an array to allow for the detection of mulliple portions of a single nucleic acid, the detection of multiple different nucleic acids, or both.

61. The method of any one of Claims 49-60 wherein the substrate is a transparent substrate or an opaque white substrate.

62. The method of Claim 61 wherein the detectable change is the formation of dark areas on the substrate.

63. The method of any one of Claims 49-60 wherein the nanoparticles in the aggregate probe are made of gold.

64. The method of any one of Claims 49-60 wherein the substrate is contacted with a silver stain to produce the detectable change.

65. The method of any one of Claims 49-60 wherein the detectable change is observed with an optical scanner.

66. A method of detecting nucleic acid having at least two portions comprising:

contacting a nucleic acid to be detected with a substrate having oligonucleotides attached thereto, the oligonucleotides having a sequence complementary to a first portion of the sequence of said nucleic acid, the contacting taking place under conditions effective to allow hybridization of the oligonucleotides on the substrate with

said nucleic acid;

contacting said nucleic acid bound to the substrate with liposomes having oligonucleotides attached thereto, the oligonucleotides having a sequence complementary to a portion of the sequence of said nucleic acid, the contacting taking place under conditions effective to allow hybridization of the oligonucleotides on the liposomes with said nucleic acid;

providing an aggregate probe comprising at least two types of nanoparticles having oligonucleotides attached thereto, the nanoparticles of the aggregate probe being bound to each other as a result of the hybridization of some of the oligonucleotides attached to them, at least one of the types of nanoparticles of the aggregate probe having oligonucleotides attached thereto which have a hydrophobic group attached to the end not attached to the nanoparticles;

contacting the liposomes bound to the substrate with the aggregate probe under conditions effective to allow attachment of the oligonucleotides on the aggregate probe to the liposomes as a result of hydrophobic interactions; and

observing a detectable change.

67. The method of Claim 66 wherein the nanoparticles in the aggregate probe are made of gold.

68. The method of Claim 66 wherein the substrate is contacted with a silver stain to produce the detectable change.

69. The method of Claim 66 wherein the substrate has a plurality of types of oligonucleotides attached to it in an array to allow for the detection of multiple portions of a single nucleic acid, the detection of multiple different nucleic acids, or both.

70. A method of detecting nucleic acid having at least two portions

comprising:

providing a substrate having oligonucleotides attached thereto, the oligonucleotides having a sequence complementary to a first portion of the sequence of a nucleic acid to be detected;

providing a core probe comprising at least two types of nanoparticles, each type of nanoparticles having oligonucleotides attached thereto which are complementary to the oligonucleotides on at least one of the other types of nanoparticles, the nanoparticles of the aggregate probe being bound to each other as a result of the hybridization of the oligonucleotides attached to them;

providing a type of nanoparticles having two types of oligonucleotides attached thereto, the first type of oligonucleotides having a sequence complementary to a second portion of the sequence of said nucleic acid, the second type of oligonucleotides having a sequence complementary to a portion of the sequence of the oligonucleotides attached to at least one of the types of nanoparticles of the core probe;

contacting said nucleic acid, the nanoparticles, the substrate and the core probe under conditions effective to allow hybridization of said nucleic acid with the oligonucleotides on the nanoparticles and with the oligonucleotides on the substrate and to allow hybridization of the oligonucleotides on the nanoparticles with the oligonucleotides on the core probe; and

observing a detectable change.

71. The method of Claim 70 wherein said nucleic acid is contacted with the substrate so that said nucleic acid hybridizes with the oligonucleotides on the substrate, and said nucleic acid bound to the substrate is then contacted with the nanoparticles so that said nucleic acid hybridizes with the oligonucleotides on the nanoparticles, and the nanoparticles bound to said nucleic acid are contacted with the core probe so that the oligonucleotides on the core probe hybridize with the oligonucleotides on the nanoparticles.

72. The method of Claim 70 wherein said nucleic acid is contacted with the nanoparticles so that said nucleic acid hybridizes with the oligonucleotides on the nanoparticles, said nucleic acid bound to the nanoparticles is then contacted with the substrate so that said nucleic acid hybridizes with the oligonucleotides on the substrate, and the nanoparticles bound to said nucleic acid are contacted with the core probe so that the oligonucleotides on the core probe hybridize with the oligonucleotides on the nanoparticles.

73. A method of detecting nucleic acid having at least two portions comprising:

providing a substrate having oligonucleotides attached thereto, the oligonucleotides having a sequence complementary to a first portion of the sequence of a nucleic acid to be detected;

providing a core probe comprising at least two types of nanoparticles, each type of nanoparticles having oligonucleotides attached thereto which are complementary to the oligonucleotides on at least one other type of nanoparticles, the nanoparticles of the aggregate probe being bound to each other as a result of the hybridization of the oligonucleotides attached to them;

providing a type of linking oligonucleotides comprising a sequence complementary to a second portion of the sequence of said nucleic acid and a sequence complementary to a portion of the sequence of the oligonucleotides attached to at least one of the types of nanoparticles of the core probe;

contacting said nucleic acid, the linking oligonucleotides, the substrate and the core probe under conditions effective to allow hybridization of said nucleic acid with the linking oligonucleotides and with the oligonucleotides on the substrate and to allow hybridization of the oligonucleotides on the linking oligonucleotides with the oligonucleotides on the core probe; and

observing a detectable change.

74. The method of any one of Claims 70-73 wherein the substrate has a plurality of types of oligonucleotides attached to it in an array to allow for the detection of multiple portions of a single nucleic acid, the detection of multiple different nucleic acids, or both.

75. The method of any one of Claims 70-73 wherein the substrate is a transparent substrate or an opaque white substrate.

76. The method of Claim 76 wherein the detectable change is the formation of dark areas on the substrate.

77. The method of any one of Claims 70-73 wherein the nanoparticles in the core probe are made of gold.

78. The method of any one of Claims 70-73 wherein the substrate is contacted with a silver stain to produce the detectable change.

79. The method of any one of Claims 70-73 wherein the detectable change is observed with an optical scanner.

80. A method of detecting a nucleic acid having at least two portions comprising:

providing nanoparticles having oligonucleotides attached thereto;

providing one or more types of binding oligonucleotides, each of the binding oligonucleotides having two portions, the sequence of one portion being complementary to the sequence of one of the portions of the nucleic acid and the sequence of the other portion being complementary to the sequence of the

oligonucleotides on the nanoparticles;

contacting the nanoparticles and the binding oligonucleotides under conditions effective to allow hybridization of the oligonucleotides on the nanoparticles with the binding oligonucleotides;

contacting the nucleic acid and the binding oligonucleotides under conditions effective to allow hybridization of the binding oligonucleotides with the nucleic acid; and

observing a detectable change.

81. The method of Claim 80 wherein the nanoparticles are contacted with the binding oligonucleotides prior to being contacted with the nucleic acid.

82. A method of detecting a nucleic acid having at least two portions comprising:

providing nanoparticles having oligonucleotides attached thereto;

providing one or more binding oligonucleotides, each of the binding oligonucleotides having two portions, the sequence of one portion being complementary to the sequence of at least two portions of the nucleic acid and the sequence of the other portion being complementary to the sequence of the oligonucleotides on the nanoparticles;

contacting the nanoparticles and the binding oligonucleotides under conditions effective to allow hybridization of the oligonucleotides on the nanoparticles with the binding oligonucleotides;

contacting the nucleic acid and the binding oligonucleotides under conditions effective to allow hybridization of the binding oligonucleotides with the nucleic acid; and

observing a detectable change.

83. A method of detecting nucleic acid having at least two portions comprising:

contacting the nucleic acid with at least two types of particles having oligonucleotides attached thereto,

the oligonucleotides on the first type of particles having a sequence complementary to a first portion of the sequence of the nucleic acid and being labeled with an energy donor,

the oligonucleotides on the second type of particles having a sequence complementary to a second portion of the sequence of the nucleic acid and being labeled with an energy acceptor,

the contacting taking place under conditions effective to allow hybridization of the oligonucleotides on the particles with the nucleic acid; and

observing a detectable change brought about by hybridization of the oligonucleotides on the particles with the nucleic acid.

84. The method of Claim 83 wherein the energy donor and acceptor are fluorescent molecules.

85. A method of detecting nucleic acid having at least two portions comprising:

providing a type of microspheres having oligonucleotides attached thereto, the oligonucleotides having a sequence complementary to a first portion of the sequence of the nucleic acid and being labeled with a fluorescent molecule;

providing a type of nanoparticles having oligonucleotides attached thereto, the oligonucleotides having a sequence complementary to a second portion of the sequence of the nucleic acid, nanoparticles being capable of producing a detectable change;

contacting the nucleic acid with the microspheres and the nanoparticles

under conditions effective to allow hybridization of the oligonucleotides on the microspheres and on the nanoparticles with the nucleic acid; and

observing a change in fluorescence, another detectable change produced by the nanoparticles, or both.

86. The method of Claim 85 wherein the detectable change produced by the nanoparticles is a change in color.

87. The method of Claim 85 wherein the microspheres are latex microspheres and the nanoparticles are gold nanoparticles, and changes in fluorescence, color or both are observed.

88. The method of Claim 87 further comprising placing a portion of the mixture of the latex microspheres, nanoparticles and nucleic acid in an observation area located on a microporous material, treating the microporous material so as to remove any unbound gold nanoparticles from the observation area, and then observing the changes in fluorescence, color, or both.

89. A method of detecting nucleic acid having at least two portions comprising:

providing a first type of metallic or semiconductor nanoparticles having oligonucleotides attached thereto, the oligonucleotides having a sequence complementary to a first portion of the sequence of the nucleic acid and being labeled with a fluorescent molecule;

providing a second type of metallic or semiconductor nanoparticles having oligonucleotides attached thereto, the oligonucleotides having a sequence complementary to a second portion of the sequence of the nucleic acid and being labeled with a fluorescent molecule;

contacting the nucleic acid with the two types of nanoparticles under conditions effective to allow hybridization of the oligonucleotides on the two types of nanoparticles with the nucleic acid; and

observing changes in fluorescence.

90. The method of Claim 89 further comprising placing a portion of the mixture of the nanoparticles and nucleic acid in an observation area located on a microporous material, treating the microporous material so as to remove any unbound nanoparticles from the observation area, and then observing the changes in fluorescence.

91. A method of detecting nucleic acid having at least two portions comprising:

providing a type of particle having oligonucleotides attached thereto, the oligonucleotides having a first portion and a second portion, both portions being complementary to portions of the sequence of the nucleic acid;

providing a type of probe oligonucleotides comprising a first portion and a second portion, the first portion having a sequence complementary to the first portion of the oligonucleotides attached to the particles and both portions being complementary to portions of the sequence of the nucleic acid, the probe oligonucleotides further being labeled with a reporter molecule at one end;

contacting the particle and the probe oligonucleotides under conditions effective to allow for hybridization of the oligonucleotides on the particles with the probe oligonucleotides to produce a satellite probe;

then contacting the satellite probe with the nucleic acid under conditions effective to provide for hybridization of the nucleic acid with the probe oligonucleotides;

removing the particles; and

detecting the reporter molecule.

92. The method of Claim 91 wherein the particles are magnetic and the reporter molecule is a fluorescent molecule.

93. The method of Claim 91 wherein the particles are magnetic and the reporter molecule is a dye molecule.

94. The method of Claim 91 wherein the particles are magnetic and the reporter molecule is a redox-active molecule.

95. A kit comprising at least one container, the container holding a composition comprising at least two types of nanoparticles having oligonucleotides attached thereto, the oligonucleotides on the first type of nanoparticles having a sequence complementary to the sequence of a first portion of a nucleic acid, the oligonucleotides on the second type of nanoparticles having a sequence complementary to the sequence of a second portion of the nucleic acid.

96. The kit of Claim 95 wherein the composition in the container further comprises a filler oligonucleotide having a sequence complementary to a third portion of the nucleic acid, the third portion being located between the first and second portions.

97. The kit of Claim 95 wherein the nanoparticles are made of gold.

98. The kit of Claim 95 further comprising a solid surface.

99. A kit comprising at least two containers,

the first container holding nanoparticles having oligonucleotides attached thereto which have a sequence complementary to the sequence of a first portion of a nucleic acid, and

the second container holding nanoparticles having oligonucleotides attached thereto which have a sequence complementary to the sequence of a second portion of the nucleic acid.

100. The kit of Claim 99 comprising a third container holding oligonucleotides having a sequence complementary to a third portion of the nucleic acid, the third portion being located between the first and second portions.

101. The kit of Claim 99 wherein the nanoparticles are made of gold.

102. The kit of Claim 99 further comprising a solid surface.

103. A kit comprising at least two containers,

the first container holding nanoparticles having oligonucleotides attached thereto which have a sequence complementary to the sequence of a first portion of a binding oligonucleotide, and

the second container holding one or more types of binding oligonucleotides, each of which has a sequence comprising at least two portions, the first portion being complementary to the sequence of the oligonucleotides on the nanoparticles and the second portion being complementary to the sequence of a portion of a nucleic acid.

104. The kit of Claim 103 which comprises additional containers, each holding an additional binding oligonucleotide, each additional binding oligonucleotide having a sequence comprising at least two portions, the first portion being complementary to the sequence of the oligonucleotides on the nanoparticles and the second portion being complementary to the sequence of another portion of the nucleic acid.

105. The kit of Claim 103 wherein the nanoparticles are made of gold.

106. The kit of Claim 103 further comprising a solid surface.

107. A kit comprising:

a container holding one type of nanoparticles having oligonucleotides attached thereto and one or more types of binding oligonucleotides, each of the types of binding oligonucleotides having a sequence comprising at least two portions, the first portion being complementary to the sequence of the oligonucleotides on the nanoparticles, whereby the binding oligonucleotides are hybridized to the oligonucleotides on the nanoparticles, and the second portion being complementary to the sequence of one or more portions of a nucleic acid.

108. A kit comprising at least one container, the container holding metallic or semiconductor nanoparticles having oligonucleotides attached thereto, the oligonucleotides having a sequence complementary to a portion of a nucleic acid and having fluorescent molecules attached to the ends of the oligonucleotides not attached to the nanoparticles.

109. A kit comprising:

a substrate, the substrate having attached thereto nanoparticles, the nanoparticles having oligonucleotides attached thereto which have a sequence complementary to the sequence of a first portion of a nucleic acid; and

a first container holding nanoparticles having oligonucleotides attached thereto which have a sequence complementary to the sequence of a second portion of the nucleic acid.

110. The kit of Claim 109 further comprising:

a second container holding a binding oligonucleotide having a selected sequence having at least two portions, the first portion being complementary to at least a portion of the sequence of the oligonucleotides on the nanoparticles in the first container; and

a third container holding nanoparticles having oligonucleotides attached thereto, the oligonucleotides having a sequence complementary to the sequence of a second portion of the binding oligonucleotide.

111. A kit comprising at least three containers:

the first container holding nanoparticles;

the second container holding a first oligonucleotide having a sequence complementary to the sequence of a first portion of a nucleic acid; and

the third container holding a second oligonucleotide having a sequence complementary to the sequence of a second portion of the nucleic acid.

112. The kit of Claim 111 further comprising a fourth container holding a third oligonucleotide having a sequence complementary to the sequence of a third portion of the nucleic acid, the third portion being located between the first and second portions.

113. The kit of Claim 111 further comprising a substrate.

114. The kit of Claim 113 further comprising:

a fourth container holding a binding oligonucleotide having a selected sequence having at least two portions, the first portion being complementary to at least a portion of the sequence of the second oligonucleotide; and

a fifth container holding an oligonucleotide having a sequence complementary to the sequence of a second portion of the binding oligonucleotide.

115. The kit of Claim 111 wherein the oligonucleotides, nanoparticles, or both bear functional groups for attachment of the oligonucleotides to the nanoparticles.

116. The kit of Claim 113 wherein the substrate, nanoparticles, or both bear functional groups for attachment of the nanoparticles to the substrate.

117. The kit of Claim 113 wherein the substrate has nanoparticles attached to it.

118. The kit of Claim 111 wherein the nanoparticles are made of gold.

119. A kit comprising:

a substrate having oligonucleotides attached thereto which have a sequence complementary to the sequence of a first portion of a nucleic acid;

a first container holding nanoparticles having oligonucleotides attached thereto, some of which have a sequence complementary to the sequence of a second portion of the nucleic acid; and

a second container holding nanoparticles having oligonucleotides attached thereto which have a sequence complementary to at least a portion of the sequence of the oligonucleotides attached to the nanoparticles in the first container.

120. A kit comprising:

a substrate;

a first container holding nanoparticles;

a second container holding a first oligonucleotide having a sequence complementary to the sequence of a first portion of a nucleic acid;

a third container holding a second oligonucleotide having a sequence complementary to the sequence of a second portion of the nucleic acid; and

a fourth container holding a third oligonucleotide having a sequence

complementary to at least a portion of the sequence of the second oligonucleotide.

121. The kit of Claim 120 wherein the oligonucleotides, nanoparticles, substrate or all bear functional groups for attachment of the oligonucleotides to the nanoparticles or for attachment of the oligonucleotides to the substrate.

122. The kit of Claim 120 wherein the nanoparticles are made of gold.

123. A kit comprising:

a substrate having oligonucleotides attached thereto which have a sequence complementary to the sequence of a first portion of a nucleic acid;

a first container holding liposomes having oligonucleotides attached thereto which have a sequence complementary to the sequence of a second portion of the nucleic acid; and

a second container holding nanoparticles having at least a first type of oligonucleotides attached thereto, the first type of oligonucleotides having a hydrophobic group attached to the end not attached to the nanoparticles.

124. The kit of Claim 123 wherein:

the nanoparticles in the second container have a second type of oligonucleotides attached thereto, the second type of oligonucleotides having a sequence complementary to the sequence of the oligonucleotides on a second type of nanoparticles; and the kit further comprises:

a third container holding a second type of nanoparticles having oligonucleotides attached thereto, the oligonucleotides having a sequence complementary to at least a portion of the sequence of the second type of oligonucleotides on the first type of nanoparticles.

125. A kit comprising:

a substrate, the substrate having attached thereto nanoparticles, the nanoparticles having oligonucleotides attached thereto which have a sequence complementary to the sequence of a first portion of a nucleic acid; and

a first container holding an aggregate probe comprising at least two types of nanoparticles having oligonucleotides attached thereto, the nanoparticles of the aggregate probe being bound to each other as a result of the hybridization of some of the oligonucleotides attached to them, at least one of the types of nanoparticles of the aggregate probe having oligonucleotides attached thereto which have a sequence complementary to a second portion of the sequence of the nucleic acid.

126. A kit comprising:

a substrate, the substrate having oligonucleotides attached thereto, the oligonucleotides having a sequence complementary to the sequence of a first portion of a nucleic acid; and

a first container holding an aggregate probe comprising at least two types of nanoparticles having oligonucleotides attached thereto, the nanoparticles of the aggregate probe being bound to each other as a result of the hybridization of some of the oligonucleotides attached to them, at least one of the types of nanoparticles of the aggregate probe having oligonucleotides attached thereto which have a sequence complementary to a second portion of the sequence of the nucleic acid.

127. The kit of Claim 126 wherein the substrate has a plurality of types of oligonucleotides attached to it in an array to allow for the detection of multiple portions of a single nucleic acid, the detection of multiple different nucleic acids, or both.

128. A kit comprising:

a substrate having oligonucleotides attached thereto;

a first container holding an aggregate probe comprising at least two types of nanoparticles having oligonucleotides attached thereto, the nanoparticles of the aggregate probe being bound to each other as a result of the hybridization of some of the oligonucleotides attached to them, at least one of the types of nanoparticles of the aggregate probe having oligonucleotides attached thereto which have a sequence complementary to a first portion of the sequence of the nucleic acid; and

a second container holding nanoparticles having at least two types of oligonucleotides attached thereto, the first type of oligonucleotides having a sequence complementary to a second portion of the sequence of the nucleic acid, and the second type of oligonucleotides having a sequence complementary to at least a portion of the sequence of the oligonucleotides attached to the substrate.

129. A kit comprising:

a substrate, the substrate having oligonucleotides attached thereto, the oligonucleotides having a sequence complementary to the sequence of a first portion of a nucleic acid;

a first container holding liposomes having oligonucleotides attached thereto which have a sequence complementary to the sequence of a second portion of the nucleic acid; and

a second container holding an aggregate probe comprising at least two types of nanoparticles having oligonucleotides attached thereto, the nanoparticles of the aggregate probe being bound to each other as a result of the hybridization of some of the oligonucleotides attached to them, at least one of the types of nanoparticles of the aggregate probe having oligonucleotides attached thereto which have a hydrophobic group attached to the end not attached to the nanoparticles.

130. The kit of any one of Claims 125-129 wherein the substrate is a transparent substrate or an opaque white substrate.

131. The kit of any one of Claims 125-129 wherein the nanoparticles of the aggregate probe are made of gold.

132. A kit comprising at least three containers:

the first container holding nanoparticles;

the second container holding a first oligonucleotide having a sequence complementary to the sequence of a first portion of a nucleic acid; and

the third container holding a second oligonucleotide having a sequence complementary to the sequence of a second portion of the nucleic acid.

133. The kit of Claim 132 further comprising a fourth container holding a third oligonucleotide having a sequence complementary to the sequence of a third portion of the nucleic acid, the third portion being located between the first and second portions.

134. The kit of Claim 132 further comprising a substrate.

135. The kit of Claim 134 further comprising:

a fourth container holding a binding oligonucleotide having a selected sequence having at least two portions, the first portion being complementary to at least a portion of the sequence of the second oligonucleotide; and

a fifth container holding an oligonucleotide having a sequence complementary to the sequence of a second portion of the binding oligonucleotide.

136. The kit of Claim 132 wherein the oligonucleotides, nanoparticles, or both bear functional groups for attachment of the oligonucleotides to the nanoparticles.

137. The kit of Claim 134 wherein the substrate, nanoparticles, or both bear

functional groups for attachment of the nanoparticles to the substrate.

138. The kit of Claim 134 wherein the substrate has nanoparticles attached to it.

139. The kit of Claim 132 wherein the nanoparticles are made of gold.

140. A kit comprising:

a substrate having oligonucleotides attached thereto which have a sequence complementary to the sequence of a first portion of a nucleic acid;

a first container holding nanoparticles having oligonucleotides attached thereto, some of which have a sequence complementary to the sequence of a second portion of the nucleic acid; and

a second container holding nanoparticles having oligonucleotides attached thereto which have a sequence complementary to at least a portion of the sequence of the oligonucleotides attached to the nanoparticles in the first container.

141. A kit comprising:

a substrate;

a first container holding nanoparticles;

a second container holding a first oligonucleotide having a sequence complementary to the sequence of a first portion of a nucleic acid;

a third container holding a second oligonucleotide having a sequence complementary to the sequence of a second portion of the nucleic acid; and

a fourth container holding a third oligonucleotide having a sequence complementary to at least a portion of the sequence of the second oligonucleotide.

142. The kit of Claim 141 wherein the oligonucleotides, nanoparticles, substrate or all bear functional groups for attachment of the oligonucleotides to the

nanoparticles or for attachment of the oligonucleotides to the substrate.

143. The kit of Claim 141 wherein the nanoparticles are made of gold.

144. A kit comprising:

a substrate having oligonucleotides attached thereto which have a sequence complementary to the sequence of a first portion of a nucleic acid;

a first container holding liposomes having oligonucleotides attached thereto which have a sequence complementary to the sequence of a second portion of the nucleic acid; and

a second container holding nanoparticles having at least a first type of oligonucleotides attached thereto, the first type of oligonucleotides having a hydrophobic group attached to the end not attached to the nanoparticles.

145. The kit of Claim 144 wherein:

the nanoparticles in the second container have a second type of oligonucleotides attached thereto, the second type of oligonucleotides having a sequence complementary to the sequence of the oligonucleotides on a second type of nanoparticles;

and the kit further comprises:

a third container holding a second type of nanoparticles having oligonucleotides attached thereto, the oligonucleotides having a sequence complementary to at least a portion of the sequence of the second type of oligonucleotides on the first type of nanoparticles.

146. A kit comprising at least two containers,

the first container holding particles having oligonucleotides attached thereto which have a sequence complementary to the sequence of a first portion of a nucleic acid, the oligonucleotides being labeled with an energy donor on the ends not

attached to the particles,

the second container holding particles having oligonucleotides attached thereto which have a sequence complementary to the sequence of a second portion of a nucleic acid, the oligonucleotides being labeled with an energy acceptor on the ends not attached to the particles.

147. The kit of Claim 146 wherein the energy donor and acceptor are fluorescent molecules.

148. A kit comprising at least one container, the container holding a first type of particles having oligonucleotides attached thereto which have a sequence complementary to the sequence of a first portion of a nucleic acid, the oligonucleotides being labeled with an energy donor on the ends not attached to the particles, and a second type of particles having oligonucleotides attached thereto which have a sequence complementary to the sequence of a second portion of a nucleic acid, the oligonucleotides being labeled with an energy acceptor on the ends not attached to the particles.

149. The kit of Claim 148 wherein the energy donor and acceptor are fluorescent molecules.

150. A kit comprising:

a first container holding a type of microspheres having oligonucleotides attached thereto, the oligonucleotides having a sequence complementary to a first portion of the sequence of a nucleic acid and being labeled with a fluorescent molecule; and

a second container holding a type of nanoparticles having oligonucleotides attached thereto, the oligonucleotides having a sequence complementary to a second portion of the sequence of the nucleic acid.

151. The kit of Claim 150 wherein the microspheres are latex microspheres and the nanoparticles are gold nanoparticles.

152. The kit of Claim 150 further comprising a microporous material.

153. A kit comprising:

a first container holding a first type of metallic or semiconductor nanoparticles having oligonucleotides attached thereto, the oligonucleotides having a sequence complementary to a first portion of the sequence of a nucleic acid and being labeled with a fluorescent molecule; and

a second container holding a second type of metallic or semiconductor nanoparticles having oligonucleotides attached thereto, the oligonucleotides having a sequence complementary to a second portion of the sequence of a nucleic acid and being labeled with a fluorescent molecule.

154. The kit of Claim 153 further comprising a microporous material.

155. A kit comprising a container holding a satellite probe, the satellite probe comprising:

a particle having attached thereto oligonucleotides, the oligonucleotides having a first portion and a second portion, both portions having sequences complementary to portions of the sequence of a nucleic acid; and

probe oligonucleotides hybridized to the oligonucleotides attached to the nanoparticles, the probe oligonucleotides having a first portion and a second portion, the first portion having a sequence complementary to the sequence of the first portion of the oligonucleotides attached to the particles, both portions having sequences complementary to portions of the sequence of the nucleic acid, the probe oligonucleotides further having a reporter molecule attached to one end.

156. A kit comprising a container holding an aggregate probe, the aggregate probe comprising at least two types of nanoparticles having oligonucleotides attached thereto, the nanoparticles of the aggregate probe being bound to each other as a result of the hybridization of some of the oligonucleotides attached to them, at least one of the types of nanoparticles of the aggregate probe having oligonucleotides attached thereto which have a sequence complementary to a portion of the sequence of a nucleic acid.

157. A kit comprising a container holding an aggregate probe, the aggregate probe comprising at least two types of nanoparticles having oligonucleotides attached thereto, the nanoparticles of the aggregate probe being bound to each other as a result of the hybridization of some of the oligonucleotides attached to them, at least one of the types of nanoparticles of the aggregate probe having oligonucleotides attached thereto which have a hydrophobic group attached to the end not attached to the nanoparticles.

158. An aggregate probe, the aggregate probe comprising at least two types of nanoparticles having oligonucleotides attached thereto, the nanoparticles of the aggregate probe being bound to each other as a result of the hybridization of some of the oligonucleotides attached to them, at least one of the types of nanoparticles of the aggregate probe having oligonucleotides attached thereto which have a sequence complementary to a portion of the sequence of a nucleic acid.

159. The aggregate probe of Claim 158 comprising two types of nanoparticles each having two types of oligonucleotides attached thereto, the first type of oligonucleotides attached to each type of nanoparticles having a sequence complementary to a portion of the sequence of a nucleic acid, the second type of oligonucleotides attached to the first type of nanoparticles having a sequence complementary to at least a portion of the sequence of the second type of oligonucleotides attached to the second type

of nanoparticles.

160. The aggregate probe of Claim 158 comprising three types of nanoparticles having oligonucleotides attached thereto, the oligonucleotides attached to the first type of nanoparticles having a sequence complementary to at least a portion of the sequence of the oligonucleotides attached to the second type of nanoparticles, the oligonucleotides attached to the second type of nanoparticles having a sequence complementary to at least a portion of the sequence of the oligonucleotides attached to the first type of nanoparticles, and the third type of nanoparticles having two types of oligonucleotides attached thereto, the first type of oligonucleotides having a sequence complementary to a portion of the sequence of a nucleic acid, and the second type of oligonucleotides having a sequence complementary to at least a portion of the sequence of the oligonucleotides attached to the first or second type of nanoparticles.

161. An aggregate probe, the aggregate probe comprising at least two types of nanoparticles having oligonucleotides attached thereto, the nanoparticles of the aggregate probe being bound to each other as a result of the hybridization of some of the oligonucleotides attached to them, at least one of the types of nanoparticles of the aggregate probe having oligonucleotides attached thereto which have a hydrophobic group attached to the end not attached to the nanoparticles.

162. A kit comprising a container holding a core probe, the core probe comprising at least two types of nanoparticles having oligonucleotides attached thereto, the nanoparticles of the core probe being bound to each other as a result of the hybridization of some of the oligonucleotides attached to them.

163. The kit of Claim 162 further comprising a substrate having oligonucleotides attached thereto, the oligonucleotides having a sequence complementary to a first portion

of the sequence of a nucleic acid to be detected.

164. The kit of Claim 162 or 163 further comprising a container holding a type of nanoparticles having two types of oligonucleotides attached thereto, the first type of oligonucleotides having a sequence complementary to a second portion of the nucleic acid, and the second type of oligonucleotides having sequence complementary to a portion of the sequence of the oligonucleotides attached to at least one of the types of nanoparticles of the core probe.

165. The kit of Claim 162 or 163 further comprising a container holding a type of linking oligonucleotides comprising a sequence complementary to a second portion of the sequence of the nucleic acid and a sequence complementary to a portion of the sequence of the oligonucleotides attached to at least one of the types of nanoparticles of the core probe.

166. A core probe comprising at least two types of nanoparticles having oligonucleotides attached thereto, the nanoparticles of the core probe being bound to each other as a result of the hybridization of some of the oligonucleotides attached to them.

167. A substrate having nanoparticles attached thereto.

168. The substrate of Claim 167 wherein the nanoparticles have oligonucleotides attached thereto which have a sequence complementary to the sequence of a first portion of a nucleic acid.

169. A metallic or semiconductor nanoparticle having oligonucleotides attached thereto, the oligonucleotides being labeled with fluorescent molecules at the ends not attached to the nanoparticle.

170. A satellite probe comprising:

a particle having attached thereto oligonucleotides, the oligonucleotides having a first portion and a second portion, both portions having sequences complementary to portions of the sequence of a nucleic acid; and

probe oligonucleotides hybridized to the oligonucleotides attached to the nanoparticles, the probe oligonucleotides having a first portion and a second portion, the first portion having a sequence complementary to the sequence of the first portion of the oligonucleotides attached to the particles, both portions having sequences complementary to portions of the sequence of the nucleic acid, the probe oligonucleotides further having a reporter molecule attached to one end.

171. A method of nanofabrication comprising

providing at least one type of linking oligonucleotide having a selected sequence, the sequence of each type of linking oligonucleotide having at least two portions;

providing one or more types of nanoparticles having oligonucleotides attached thereto, the oligonucleotides on each of the types of nanoparticles having a sequence complementary to the sequence of a portion of a linking oligonucleotide; and

contacting the linking oligonucleotides and nanoparticles under conditions effective to allow hybridization of the oligonucleotides on the nanoparticles to the linking oligonucleotides so that a desired nanomaterial or nanostructure is formed wherein the nanoparticles are held together by oligonucleotide connectors.

172. The method of Claim 171 wherein at least two types of nanoparticles having oligonucleotides attached thereto are provided, the oligonucleotides on the first type of nanoparticles having a sequence complementary to a first portion of the sequence of a linking oligonucleotide, and the oligonucleotides on the second type of nanoparticles

having a sequence complementary to a second portion of the sequence of the linking oligonucleotide.

173. The method of Claim 171 or 172 wherein the nanoparticles are metallic nanoparticles, semiconductor nanoparticles, or a combination thereof.

174. The method of Claim 173 wherein the metallic nanoparticles are made of gold, and the semiconductor nanoparticles are made of CdSe/ZnS (core/shell).

175. A method of nanofabrication comprising:

providing at least two types of nanoparticles having oligonucleotides attached thereto,

the oligonucleotides on the first type of nanoparticles having a sequence complementary to that of the oligonucleotides on the second of the nanoparticles;

the oligonucleotides on the second type of nanoparticles having a sequence complementary to that of the oligonucleotides on the first type of nanoparticles; and

contacting the first and second types of nanoparticles under conditions effective to allow hybridization of the oligonucleotides on the nanoparticles to each other so that a desired nanomaterial or nanostructure is formed.

176. The method of Claim 175 wherein the nanoparticles are metallic nanoparticles, semiconductor nanoparticles, or a combination thereof.

177. The method of Claim 176 wherein the metallic nanoparticles are made of gold, and the semiconductor nanoparticles are made of CdSe/ZnS (core/shell).

178. Nanomaterials or nanostructures composed of nanoparticles having

oligonucleotides attached thereto, the nanoparticles being held together by oligonucleotide connectors.

179. The nanomaterials or nanostructures of Claim 178 wherein at least some of the oligonucleotide connectors are triple-stranded.

180. The nanomaterials or nanostructures of Claim 178 wherein the nanoparticles are metallic nanoparticles, semiconductor nanoparticles, or a combination thereof.

181. The nanomaterials or nanostructures of Claim 180 wherein the metallic nanoparticles are made of gold, and the semiconductor nanoparticles are made of CdSe/ZnS (core/shell).

182. A composition comprising at least two types of nanoparticles having oligonucleotides attached thereto, the oligonucleotides on the first type of nanoparticles having a sequence complementary to the sequence of a first portion of a nucleic acid or a linking oligonucleotide, the oligonucleotides on the second type of nanoparticles having a sequence complementary to the sequence of a second portion of the nucleic acid or linking oligonucleotide.

183. The composition of Claim 182 wherein the nanoparticles are metallic nanoparticles, semiconductor nanoparticles, or a combination thereof.

184. The composition of Claim 183 wherein the metallic nanoparticles are made of gold, and the semiconductor nanoparticles are made of CdSe/ZnS (core/shell).

185. An assembly of containers comprising:

a first container holding nanoparticles having oligonucleotides attached thereto, and

a second container holding nanoparticles having oligonucleotides attached thereto,

the oligonucleotides attached to the nanoparticles in the first container having a sequence complementary to that of the oligonucleotides attached to the nanoparticles in the second container,

the oligonucleotides attached to the nanoparticles in the second container having a sequence complementary to that of the oligonucleotides attached to the nanoparticles in the second container.

186. The assembly of Claim 185 wherein the nanoparticles are metallic nanoparticles, semiconductor nanoparticles, or a combination thereof.

187. The assembly of Claim 186 wherein the metallic nanoparticles are made of gold, and the semiconductor nanoparticles are made of CdSe/ZnS (core/shell).

188. A nanoparticle having a plurality of different oligonucleotides attached thereto.

189. A method of separating a selected nucleic acid having at least two portions from other nucleic acids, the method comprising:

providing two or more types of nanoparticles having oligonucleotides attached thereto, the oligonucleotides on each of the types of nanoparticles having a sequence complementary to the sequence of one of the portions of the selected nucleic acid; and

contacting the nucleic acids and nanoparticles under conditions effective to allow hybridization of the oligonucleotides on the nanoparticles with the selected

nucleic acid so that the nanoparticles hybridized to the selected nucleic acid aggregate and precipitate.

190. A method of binding oligonucleotides to charged nanoparticles to produce stable nanoparticle-oligonucleotide conjugates, the method comprising:

providing oligonucleotides having covalently bound thereto a moiety comprising a functional group which can bind to the nanoparticles;

contacting the oligonucleotides and the nanoparticles in water for a period of time sufficient to allow at least some of the oligonucleotides to bind to the nanoparticles;

adding at least one salt to the water to form a salt solution, the ionic strength of the salt solution being sufficient to overcome at least partially the electrostatic attraction or repulsion of the oligonucleotides for the nanoparticles and the electrostatic repulsion of the oligonucleotides for each other; and

contacting the oligonucleotides and nanoparticles in the salt solution for an additional period of time sufficient to allow sufficient additional oligonucleotides to bind to the nanoparticles to produce the stable nanoparticle-oligonucleotide conjugates.

191. The method of Claim 190 wherein the nanoparticles are metal nanoparticles or semiconductor nanoparticles.

192. The method of Claim 191 wherein the nanoparticles are gold nanoparticles.

193. The method of Claim 192 wherein the moiety comprising a functional group which can bind to the nanoparticles is an alkanethiol.

194. The method of Claim 190 wherein all of the salt is added to the water in a

single addition.

195. The method of Claim 190 wherein the salt is added gradually over time.

196. The method of Claim 190 wherein the salt is selected from the group consisting of sodium chloride, maguesium chloride, potassium chloride, ammonium, chloride, sodium, acetate, ammonium acetate, a combination of two or more of these salts, one of these salts in a phosphate buffer, and a combination of two or more these salts in a phosphate buffer.

197. The method of Claim 196 wherein the salt is sodium chloride in a phosphate buffer.

198. The method of Claim 190 wherein nanoparticle-oligonucleotide conjugates are produced which have the oligonucleotides present on surface of the nanoparticles at a surface density of at least 10 picomoles/cm^2.

199. The method of Claim 198 wherein the oligonucleotides are present on surface of the nanoparticles at a surface density of at least 15 picomoles/cm^2.

200. The method of Claim 199 wherein the oligonucleotides are present on surface of the nanoparticles at a surface density of from about 15 picomoles/cm^2 to about 40 picomoles/cm^2.

201. A method of binding oligonucleotides to nanoparticles to produce nanoparticle-oligonucleotide conjugates, the method comprising:

providing oligonucleotides, the oligonucleotides comprising at least one type of recognition oligonucleotides, each of the recognition oligonucleotides comprising

FIG.1

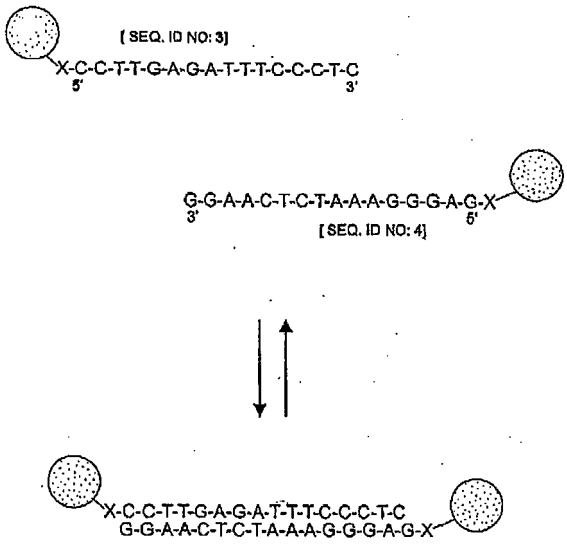

[SEQ. ID NO: 3]
X-C-C-T-T-G-A-G-A-T-T-T-C-C-C-T-C
5'
3'
G-G-A-A-C-T-C-T-A-A-A-G-G-G-A-G-X
3'
5'
[SEQ. ID NO: 4]
X-C-C-T-T-G-A-G-A-T-T-T-C-C-C-T-C
G-G-A-A-C-T-C-T-A-A-A-G-G-G-A-G-X

FIG.2

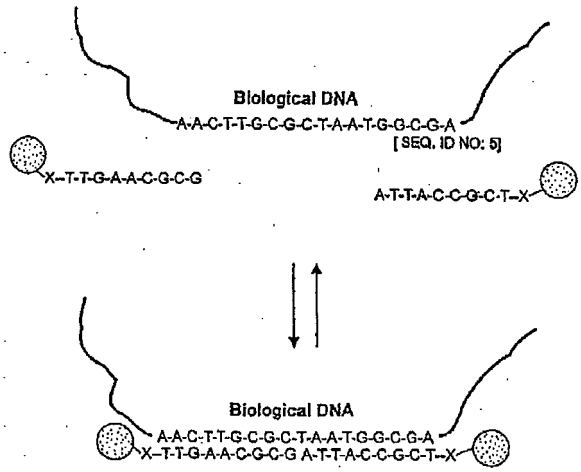

Biological DNA
A-A-C-T-T-G-C-G-C-T-A-A-T-G-G-C-G-A
[SEQ. ID NO: 5]
X–T-T-G-A-A-C-G-C-G
A-T-T-A-C-C-G-C-T–X
Biological DNA
A-A-C-T-T-G-C-G-C-T-A-A-T-G-G-C-G-A
X–T-T-G-A-A-C-G-C-G A-T-T-A-C-C-G-C-T–X

FIG.3

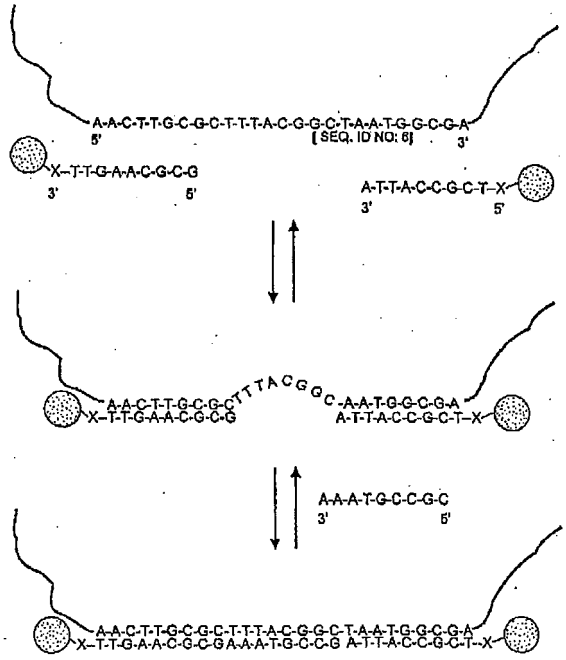

A-A-C-T-T-G-C-G-C-T-T-T-A-C-G-G-C-T-A-A-T-G-G-C-G-A
5'
[SEQ. ID NO: 6]
3'
X–T-T-G-A-A-C-G-C-G
3'
5'
A-T-T-A-C-C-G-C-T–X
3'
5'
A-A-C-T-T-G-C-G-C
T T A C G G C
A-A-T-G-G-C-G-A
X–T-T-G-A-A-C-G-C-G
A-T-T-A-C-C-G-C-T–X
A-A-A-T-G-C-C-G-C
3'
5'
A-A-C-T-T-G-C-G-C-T-T-T-A-C-G-G-C-T-A-A-T-G-G-C-G-A
X–T-T-G-A-A-C-G-C-G A-A-A-T-G-C-C-G A-T-T-A-C-C-G-C-T–X

FIG.4

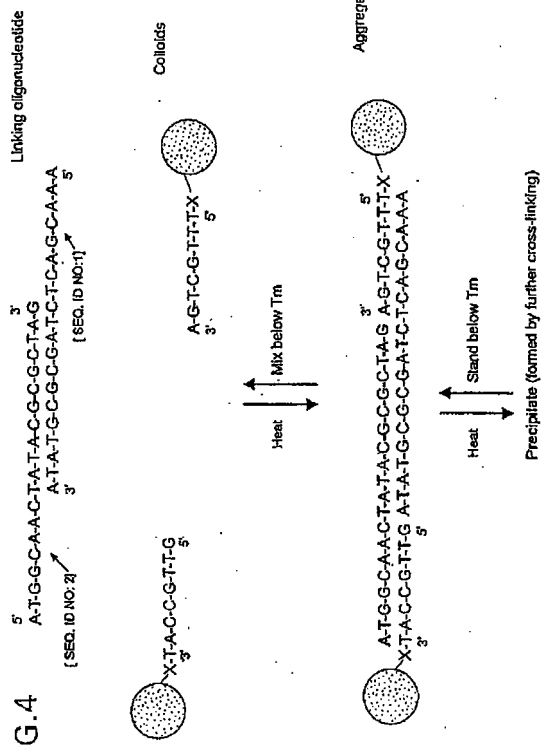

Linking oligonucleotide
5' A-T-G-G-C-A-A-C-T-A-T-A-C-G-C-G-C-T-A-G 3'
[SEQ. ID NO: 2]
3' A-T-A-T-G-C-G-C-G-A-T-C-T-C-A-G-C-A-A-A 5'
[SEQ. ID NO:1]
Colloids
X-T-A-C-C-G-T-T-G 5'
3'
A-G-T-C-G-T-T-T-X 5'
3'
Heat
Mix below Tm
Aggregate
A-T-G-G-C-A-A-C-T-A-T-A-C-G-C-G-C-T-A-G 3'
X-T-A-C-C-G-T-T-G A-T-A-T-G-C-G-C-G-A-T-C-T-C-A-G-C-A-A-A 5'
3' A-G-T-C-G-T-T-T-X 5'
Heat
Stand below Tm
Precipitate (formed by further cross-linking)

FIG.5

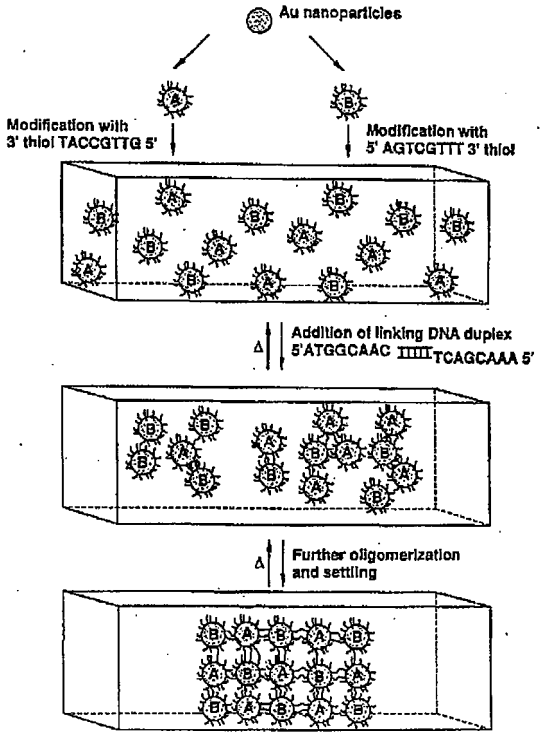

Au nanoparticles
Modification with
3' thiol TACCGTTG 5'
Modification with
5' AGTCGTTT 3' thiol
Δ
Addition of linking DNA duplex
5'ATGGCAAC IIIII TCAGCAAA 5'
Δ
Further oligomerization
and settling

FIG.6A FIG.6B FIG.6C

FIG. 7

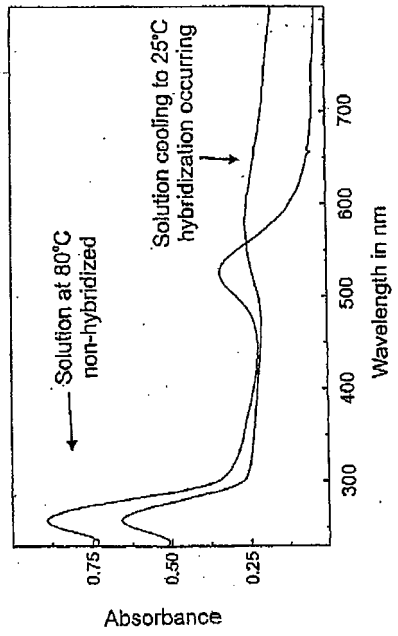

Solution at 80°C
non-hybridized
Solution cooling to 25°C
hybridization occurring
0.75
0.50
0.25
Absorbance
300
400
500
600
700
Wavelength in nm

FIG.8B

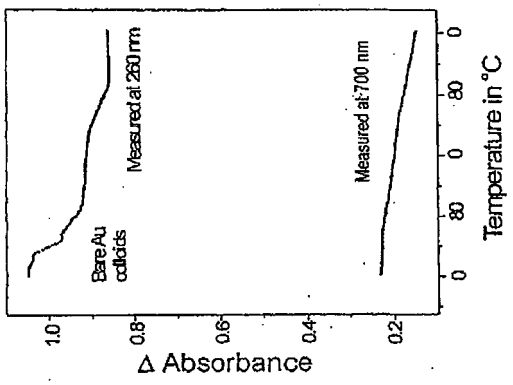

Bare Au colloids
Measured at 260 nm
Measured at 700 nm
Δ Absorbance
Temperature in °C

FIG.8A

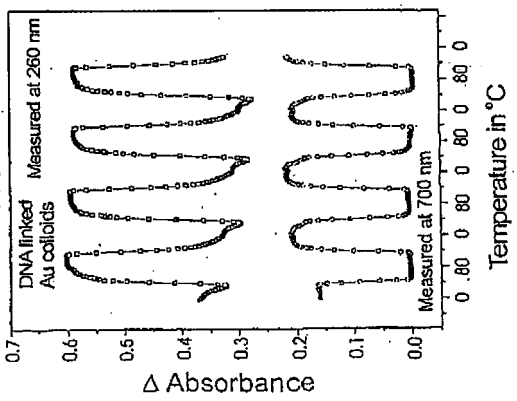

DNA linked Au colloids
Measured at 260 nm
Measured at 700 nm
Δ Absorbance
Temperature in °C

FIG.9A

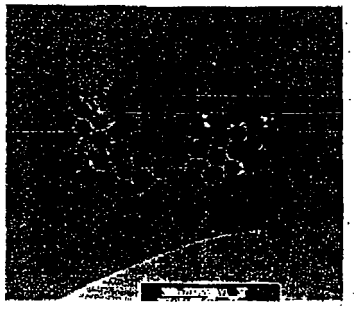


FIG.9B

FIG.10

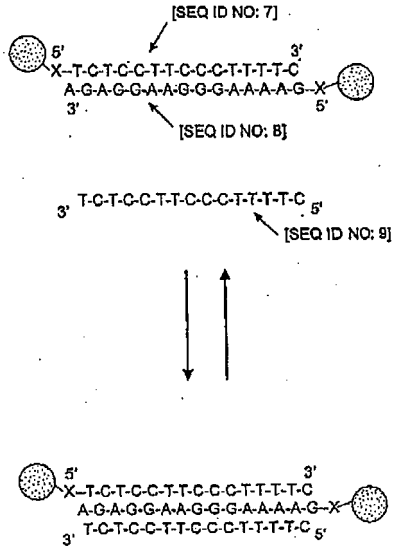

[SEQ ID NO: 7]
5'
3'
X–T-C-T-C-C-T-T-C-C-C-T-T-T-T-C
A-G-A-G-G-A-A-G-G-G-A-A-A-A-G–X
3'
5'
[SEQ ID NO: 8]
3' T-C-T-C-C-T-T-C-C-C-T-T-T-T-C 5'
[SEQ ID NO: 9]
5'
3'
X–T-C-T-C-C-T-T-C-C-C-T-T-T-T-C
A-G-A-G-G-A-A-G-G-G-A-A-A-A-G–X
3' T-C-T-C-C-T-T-C-C-C-T-T-T-T-C 5'

FIG.11

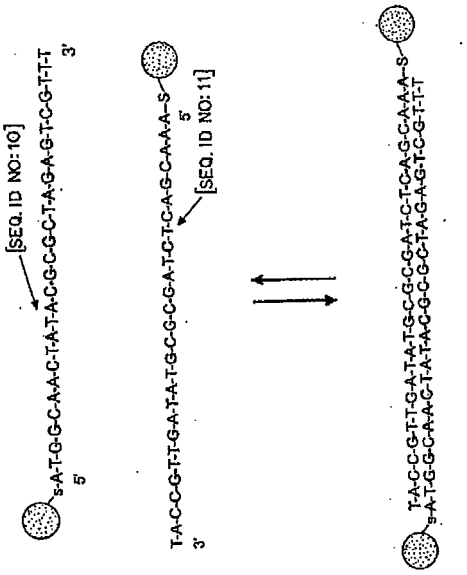

[SEQ. ID NO: 10]
s-A-T-G-C-A-A-C-T-A-T-A-C-G-C-G-C-T-A-G-A-G-T-C-G-T-T-T
5'
3'
T-A-C-G-T-T-G-A-T-A-T-G-C-G-C-G-A-T-C-T-C-A-G-C-A-A-A-s
3'
5'
[SEQ. ID NO: 11]
T-A-C-G-T-T-G-A-T-A-T-G-C-G-C-G-A-T-C-T-C-A-G-C-A-A-A-s
s-A-T-G-C-A-A-C-T-A-T-A-C-G-C-G-C-T-A-G-A-G-T-C-G-T-T-T

FIG.12A
Complementary Target

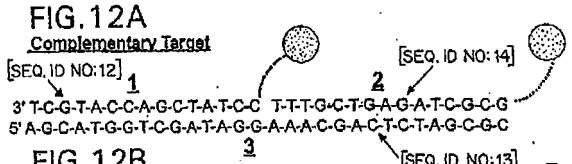


FIG.12B
Probes without Target

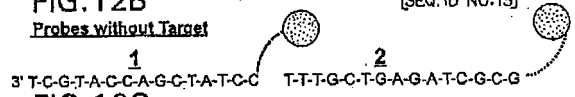


FIG.12C
Half Complementary Target

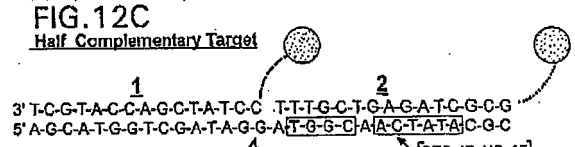


FIG.12D
Target - 6 bp

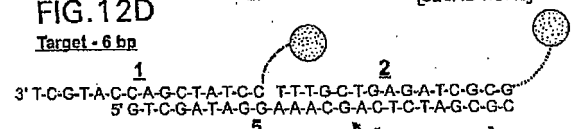


FIG.12E
One bp Mismatch

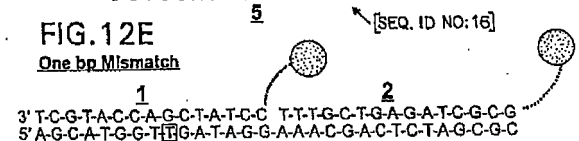


FIG.12F
Two bp Mismatch

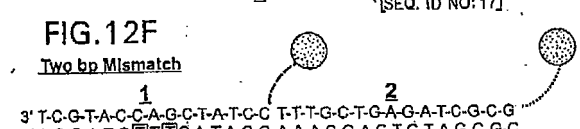


FIG.13A

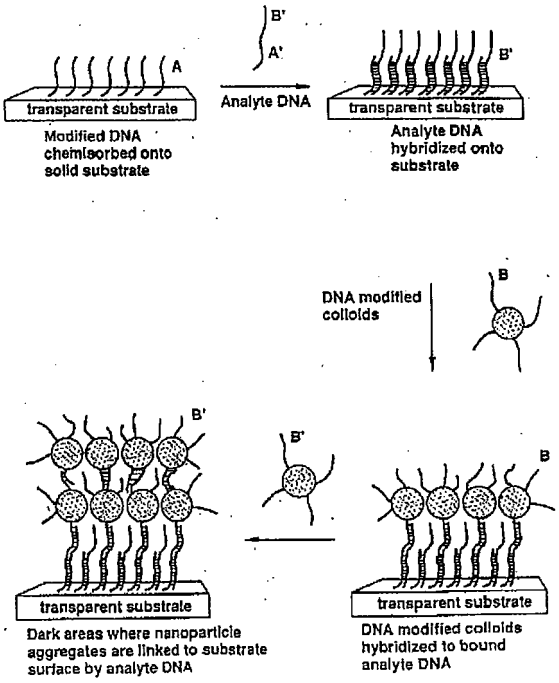

B'
A'
A
transparent substrate
Analyte DNA
B'
transparent substrate
Modified DNA chemisorbed onto solid substrate
Analyte DNA hybridized onto substrate
DNA modified colloids
B
B'
B'
B
transparent substrate
transparent substrate
Dark areas where nanoparticle aggregates are linked to substrate surface by analyte DNA
DNA modified colloids hybridized to bound analyte DNA

FIG.13B

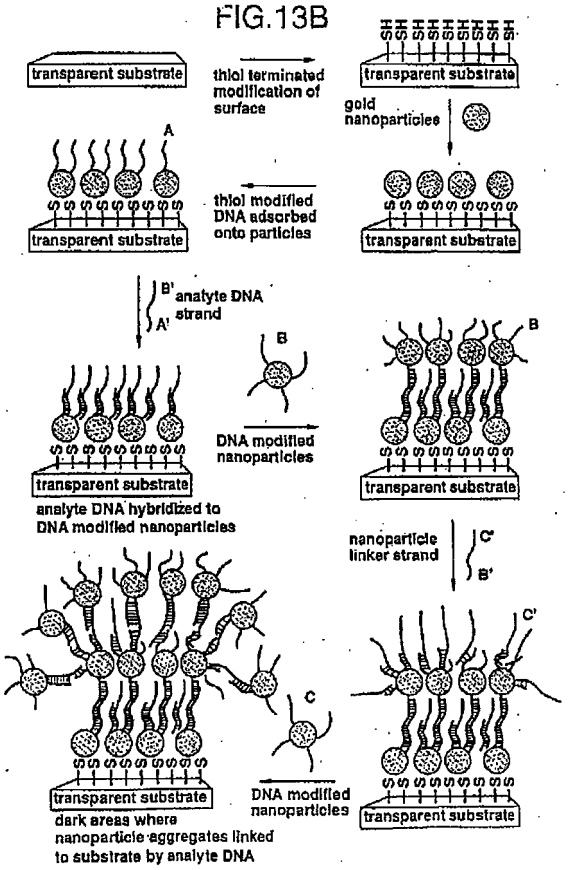

transparent substrate
thiol terminated modification of surface
transparent substrate
gold nanoparticles
A
thiol modified DNA adsorbed onto particles
transparent substrate
transparent substrate
B'
A'
analyte DNA strand
B
DNA modified nanoparticles
B
transparent substrate
transparent substrate
analyte DNA hybridized to DNA modified nanoparticles
nanoparticle linker strand
C'
B'
C'
C
transparent substrate
DNA modified nanoparticles
transparent substrate
dark areas where nanoparticle aggregates linked to substrate by analyte DNA

FIG.14A

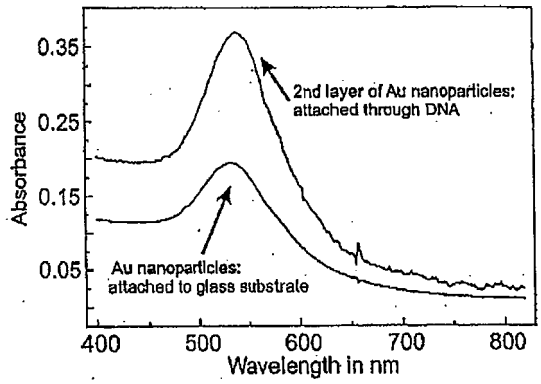


FIG.14B

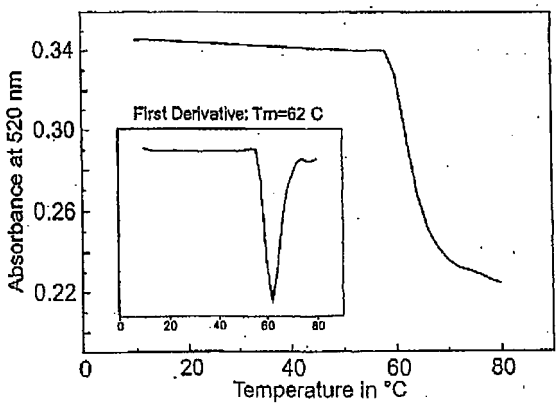


FIG15A
Probes with No Target

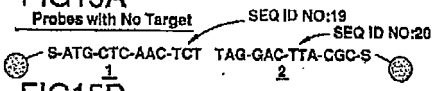


FIG15B
Half-Complementary Target

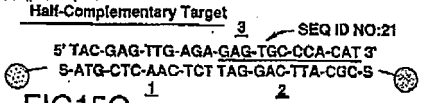


FIG15C
Complementary Target Tm=53.5°C

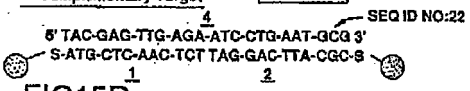


FIG15D
ONE Base-Pair Mismatch at Probe Head Tm=50.4°C

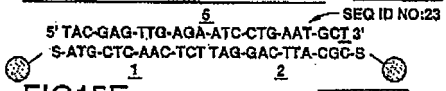


FIG15E
ONE Base-Pair Mismatch at Probe Tail Tm=46.2°C

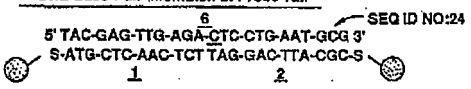


FIG15F
ONE Base Deletion Tm=51.6°C

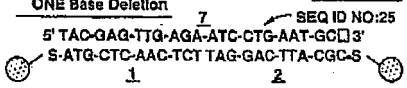


FIG15G
ONE Base-Pair Insertion Tm=50.2°C

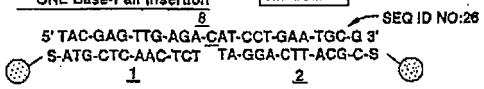


FIG.16A 24 Base Template

5' TAC-GAG-TTG-AGA-ATC-CTG-AAT-GCG 3'
S-ATG-CTC-AAC-TCT TAG-GAC-TTA-CGC-S
1 2

FIG.16B 48 Base Template with Complementary 24 Base Filler

5' TAC-GAG-TTG-AGA-CCG-TTA-AGA-CGA-GGC-AAT-CAT-GCA-ATC-CTG-AAT-GCG 3'
S-ATG-CTC-AAC-TCT GGC-AAT-TCT-GCT-CCG-TTA-GTA-CGT TAG-GAC-TTA-CGC-S
1 2

FIG.16C 72 Base Template with Complementary 48 Base Filler

5' TAC-GAG-TTG-AGA-CCG-TTA-AGA-CGA-GGC-AAT-CAT-GCA-TAT-ATT-GGA-CGC-TTT-ACG-GAC-AAC-ATC-CTG-AAT-GCG 3'
S-ATG-CTC-AAC-TCT GGC-AAT-TCT-GCT-CCG-TTA-GTA-CGT-ATA-TAA-CCT-GCG-AAA-TGC-CTG-TTG TAG-GAC-TTA-CGC-S
1 2

FIG.17A

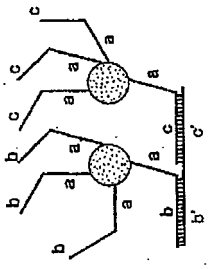


FIG.17B

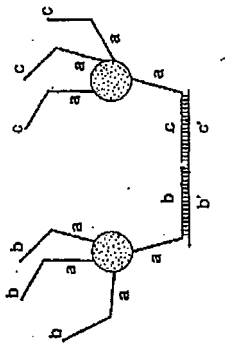


FIG.17C

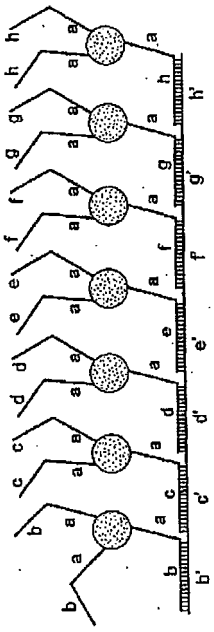


FIG.17D

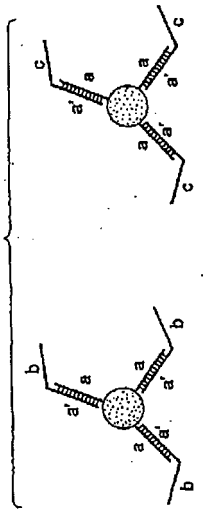


FIG.17E

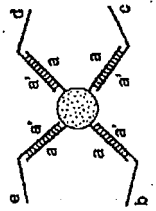


FIG.18

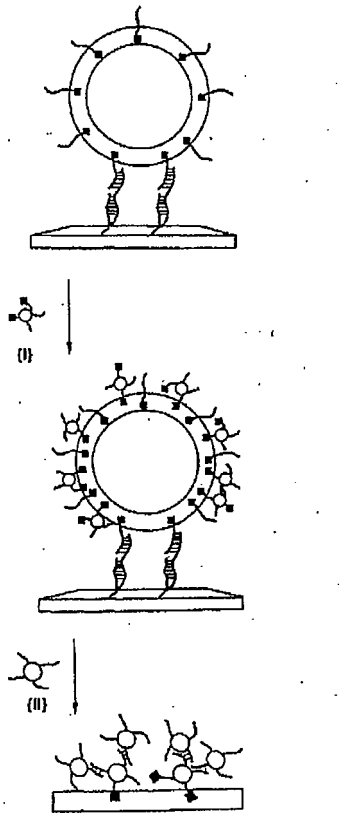


FIG.19A

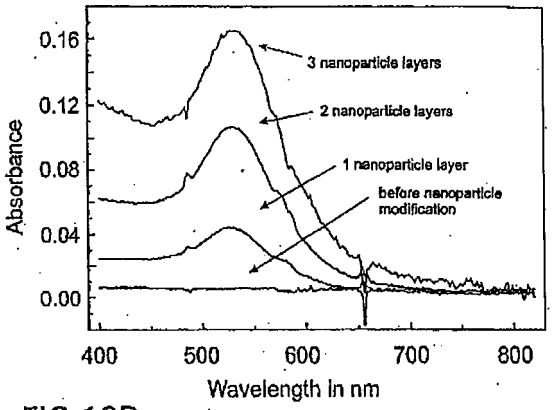


FIG.19B

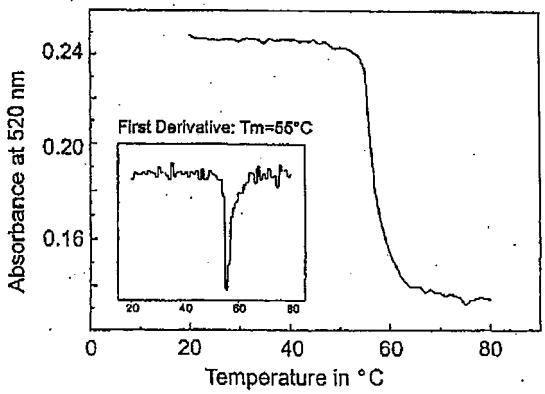


FIG.20A

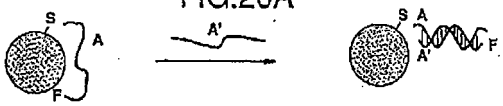
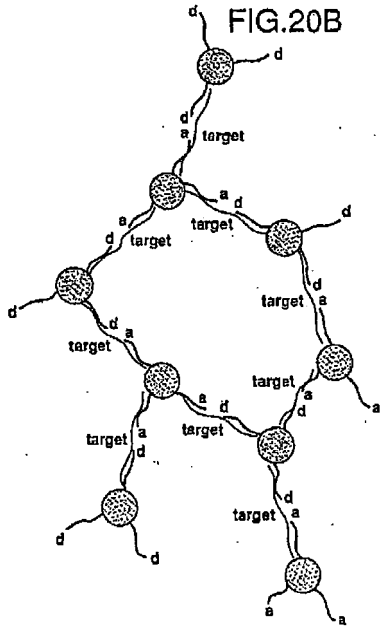


FIG.20B



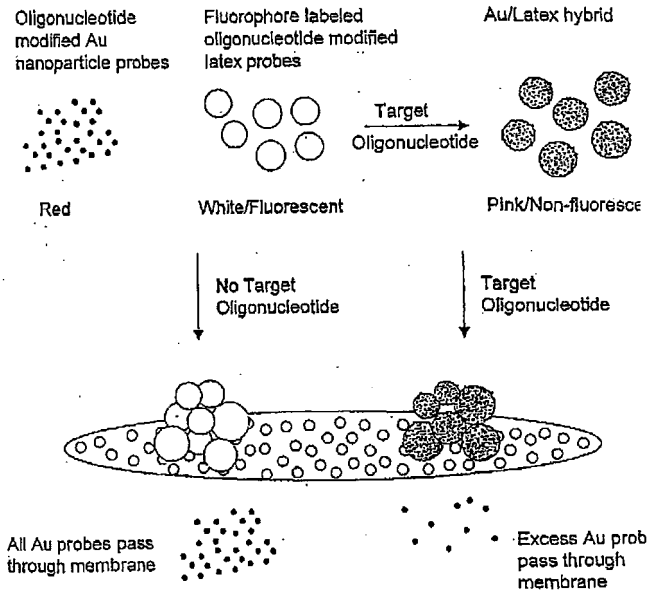
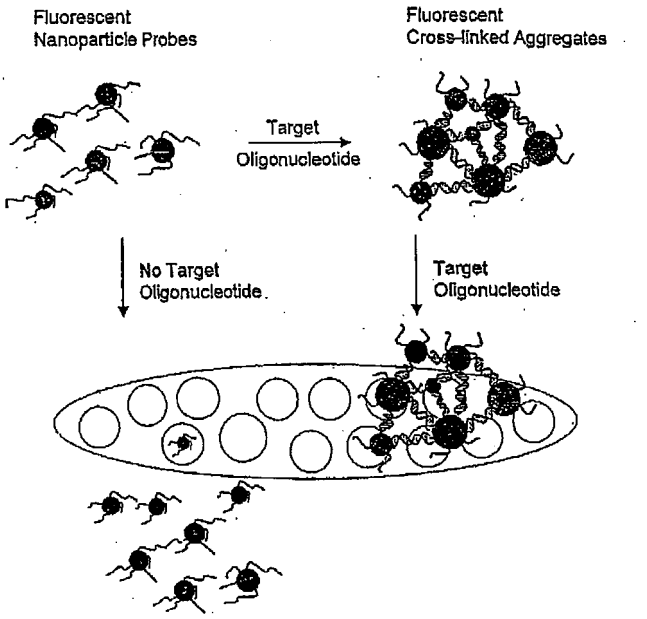

Oligonucleotide modified Au nanoparticle probes
Fluorophore labeled oligonucleotide modified latex probes
Au/Latex hybrid
Target Oligonucleotide
Red
White/Fluorescent
Pink/Non-fluoresce
No Target Oligonucleotide
Target Oligonucleotide
All Au probes pass through membrane
Excess Au prob pass through membrane

FIGURE 21

FIGURE 22



The fluorescent nanoparticle probes pass through the membrane

The fluorescent cross-linked aggregate are retained by the membrane

FIG. 23

Anthrax PCR Product

5'G GCG GAT GAG TCA GTA GTT AAG GAG GCT CAT AGA GAA GTA ATT AAT
3'C CGC CTA CTC AGT CAT CAA TTC CTC CGA GTA TCT CTT CAT TAA TTA

TCG TCA ACA GAG GGA TTA TTG TTA AAT ATT GAT AAG GAT ATA AGA AAA
AGC AGT TGT CTC CCT AAT AAC AAT TTA TAA CTA TTC CTA TAT TCT TTT

ATA TTA TCC AGG GTT ATA TTG TAG AAA TTG AAG ATA CTG AAG GGC TT 3'
TAT AAT AGG TCC CAA TAT AAC ATC TTT AAC TTC TAT GAC TTC CCG AA 5'

141 mer Anthrax PCR product [SEQ ID NO:36]

3' CTC CCT AAT AAC AAT

[SEQ ID NO:37]

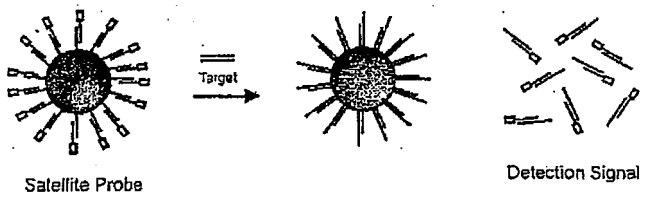
3' TTA TAA CTA TTC CTA

[SEQ ID NO:38]

Oligonucleotide-Nanoparticle Probes

Blocker Oligonucleotides

3' C CGC CTA CTC AGT CAT CAA TTC CTC CGA GT	[SEQ ID NO:39]
3' A TCT CTT CAT TAA TTA AGC AGT TGT	[SEQ ID NO:40]
3' TAT TCT TTT TAT AAT AGG TCC CAA TAT	[SEQ ID NO:41]
3' AAC ATC TTT AAC TTC TAT GAC TTC CCG AA	[SEQ ID NO:42]


Target
Satellite Probe
Detection Signal

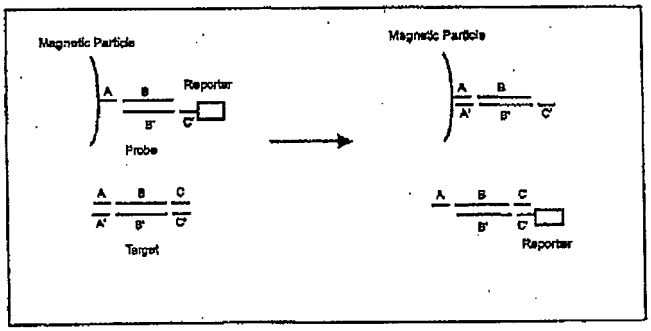

Magnetic Particle
A
B
Reporter
B'
C'
Probe
A
B
C
A'
B'
C'
Target
Magnetic Particle
A
B
A'
B'
C'
A
B
C
B'
C'
Reporter

FIGURE 24

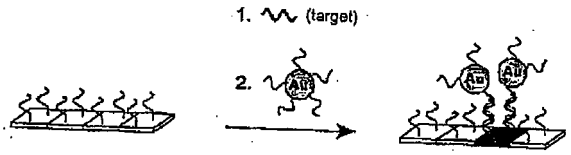


FIGURE 25A

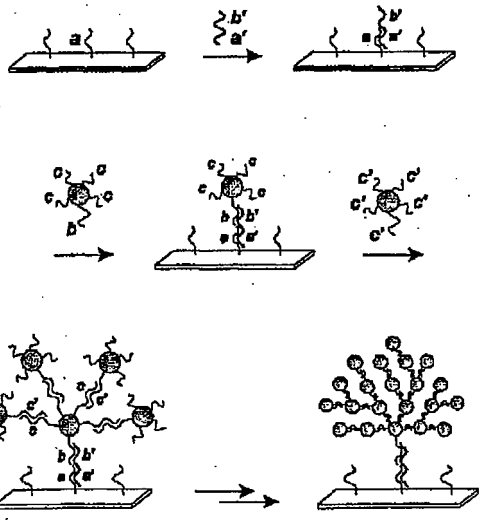


FIGURE 25B

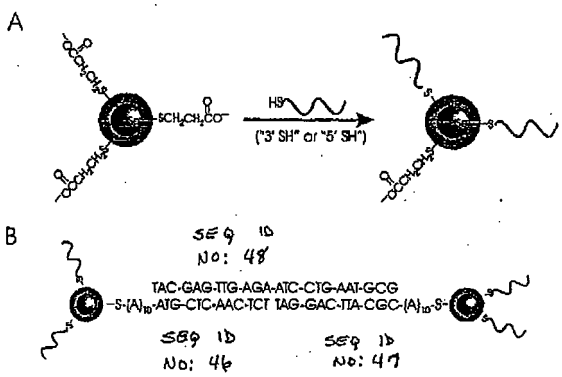

A
HS
("3' SH" or "5' SH")
B
SEQ ID NO: 48
TAC-GAG-TTG-AGA-ATC-CTG-AAT-GCG
ATG-CTC-AAC-TCT TAG-GAC-TTA-CGC-
SEQ ID NO: 46
SEQ ID NO: 47

FIGURE 26

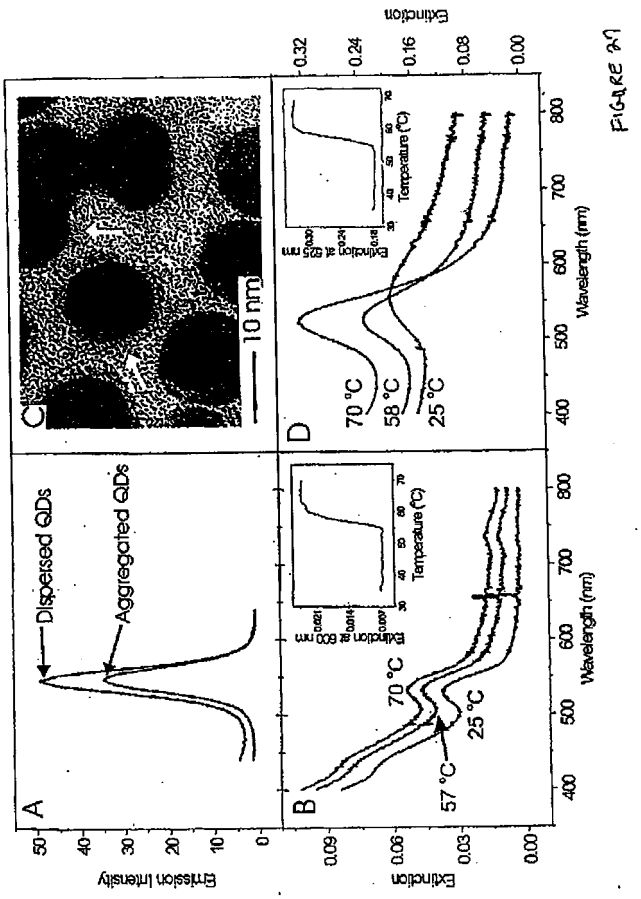

A
Dispersed QDs
Aggregated QDs
Emission Intensity
Wavelength (nm)
B
Extinction
Temperature (°C)
57 °C
70 °C
25 °C
C
10 nm
D
70 °C
58 °C
25 °C

FIGURE 27

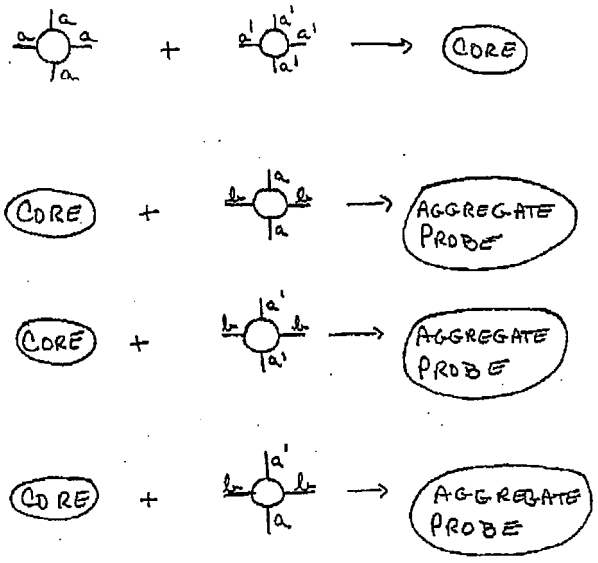

a
a
a
a
a'
a'
a'
a'
CORE
CORE
AGGREGATE PROBE
CORE
AGGREGATE PROBE
CORE
AGGREGATE PROBE

FIGURE 29A

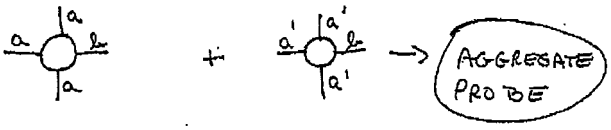


FIGURE 88 B

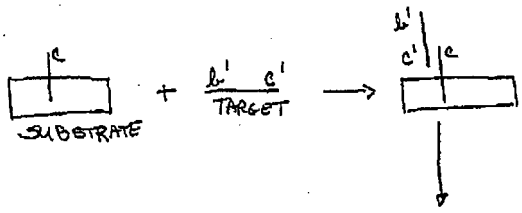


FIG. 28C

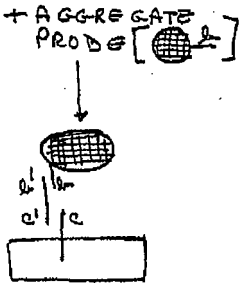


FIG. 28D

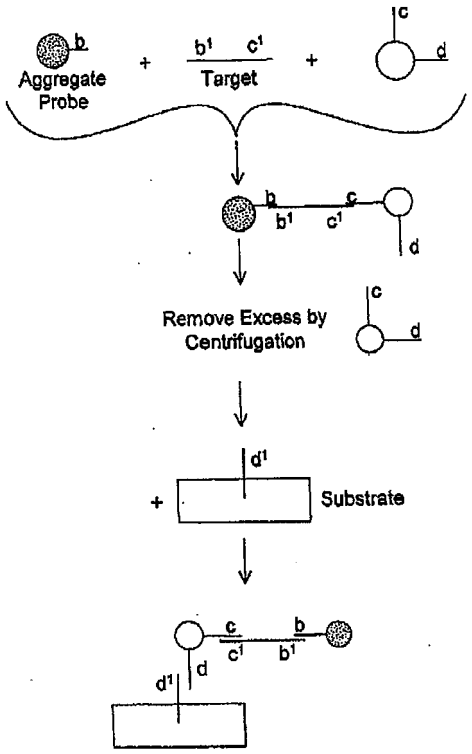
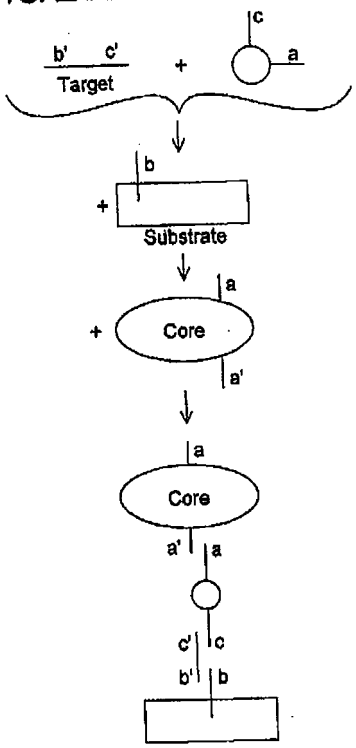

b
Aggregate Probe
+
b¹ c¹
Target
+
c
d
b
b¹
c¹
c
d
Remove Excess by Centrifugation
c
d
d¹
+
Substrate
c
b
c¹
b¹
d
d¹

FIG. 28E



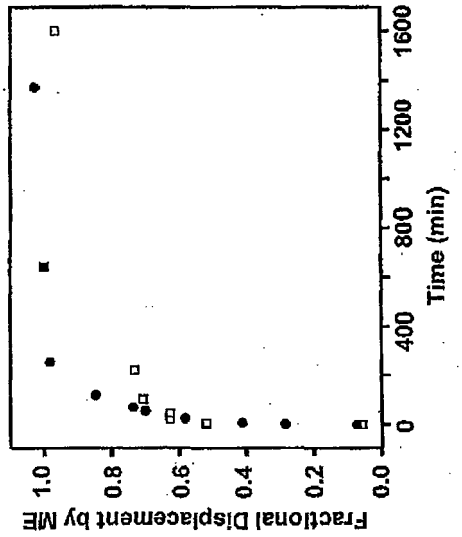

Fractional Displacement by ME
0.0
0.2
0.4
0.6
0.8
1.0
0
400
800
1200
1600
Time (min)

Figure 29

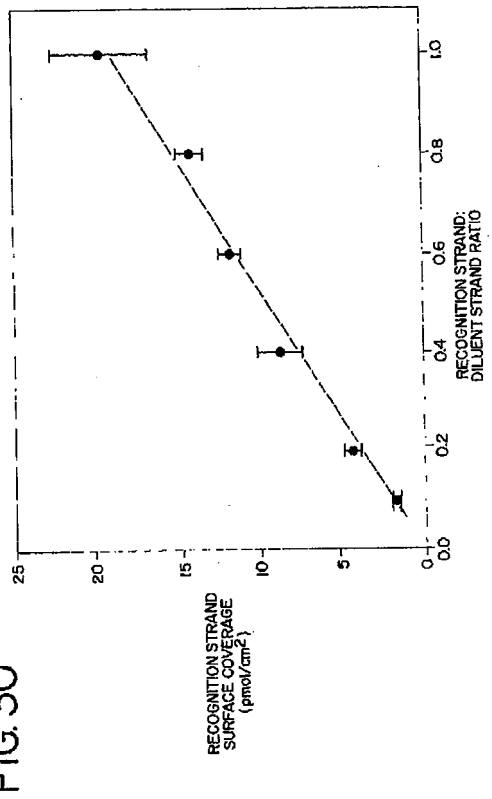

RECOGNITION STRAND SURFACE COVERAGE (pmol/cm²)
0
5
10
15
20
25
RECOGNITION STRAND: DILUENT STRAND RATIO
0.0
0.2
0.4
0.6
0.8
1.0

FIG. 30

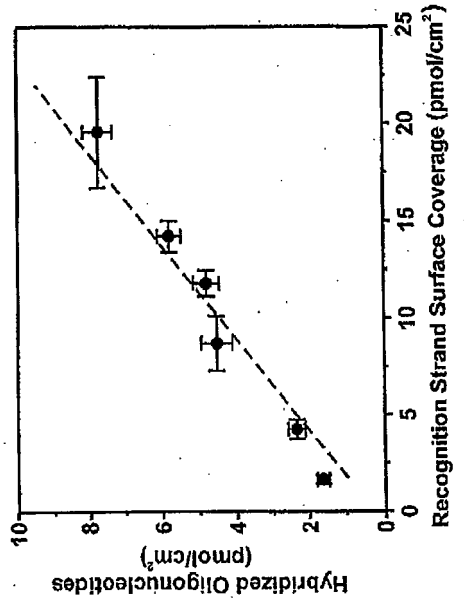
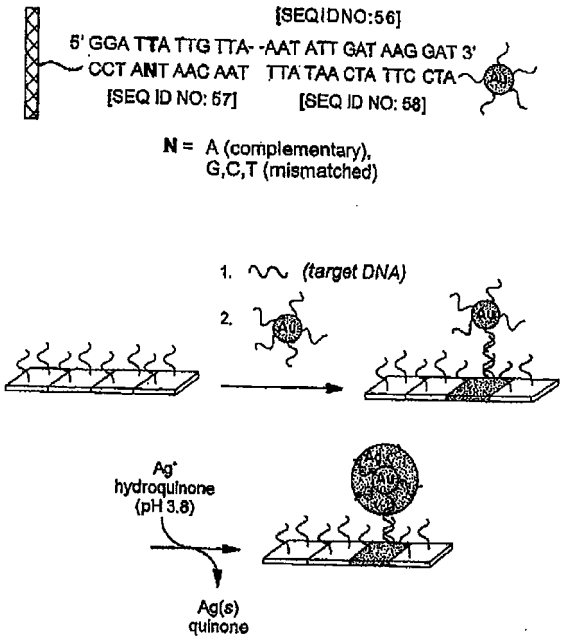

Hybridized Oligonucleotides (pmol/cm²)
0
2
4
6
8
10
Recognition Strand Surface Coverage (pmol/cm²)
0
5
10
15
20
25

Figure 31

FIG. 32


[SEQ ID NO:56]
5' GGA TTA TTG TTA- -AAT ATT GAT AAG GAT 3'
CCT ANT AAC AAT TTA TAA CTA TTC CTA
[SEQ ID NO: 57]
[SEQ ID NO: 58]
N = A (complementary), G,C,T (mismatched)
1. (target DNA)
2.
Ag+ hydroquinone (pH 3.8)
Ag(s) quinone

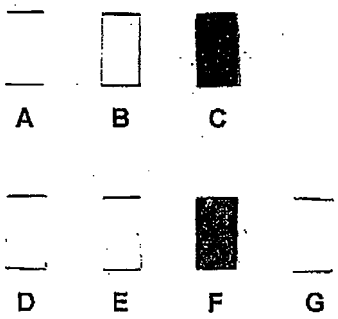


Figure 33

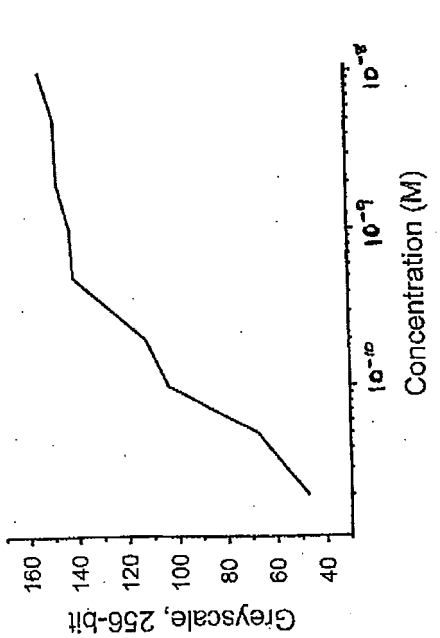

Greyscale, 256-bit
160
140
120
100
80
60
40
Concentration (M)
10^-10
10^-9
10^-8

Figure 34

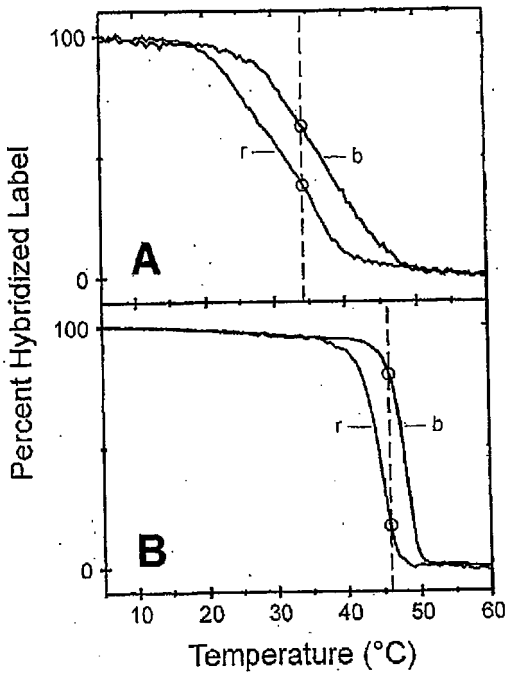

100
0
r
b
A
100
0
r
b
B
Percent Hybridized Label
10
20
30
40
50
60
Temperature (°C)

Figure 35

FIG. 36A

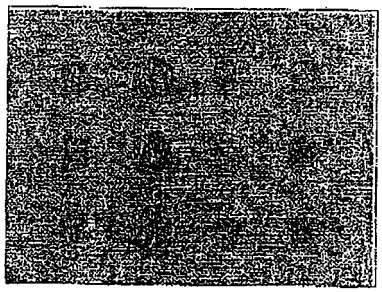
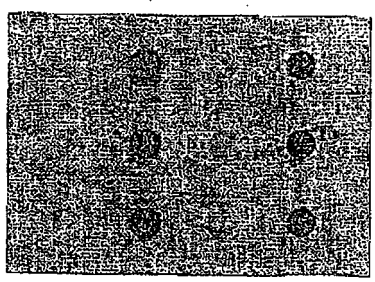


FIG. 36B



C A T G

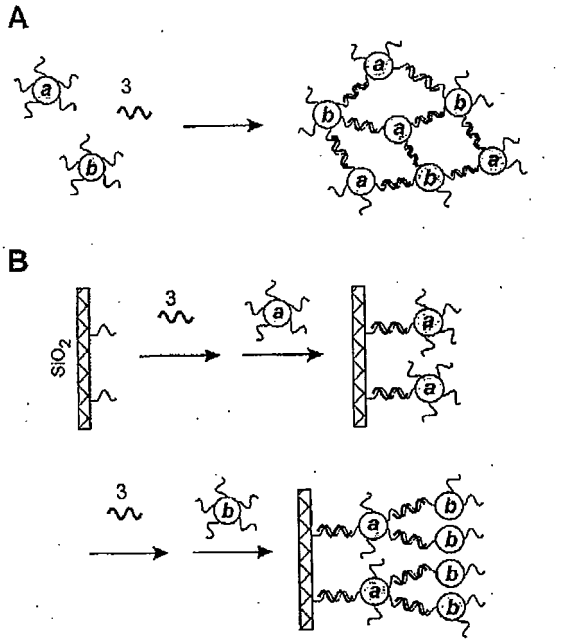


Figure 37

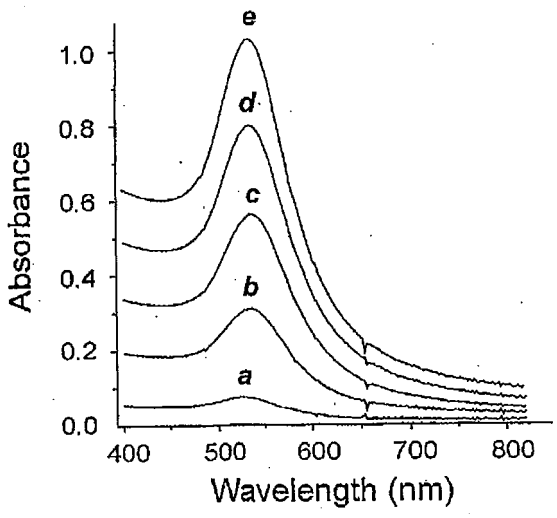

e
d
c
b
a
1.0
0.8
0.6
0.4
0.2
0.0
Absorbance
400
500
600
700
800
Wavelength (nm)

Figure 38A

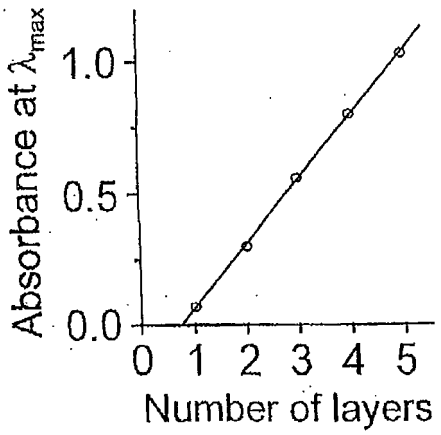


Figure 38B

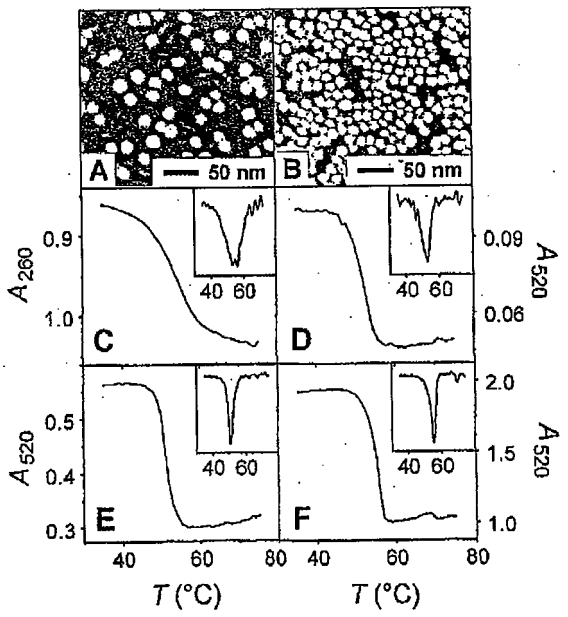

A
50 nm
B
50 nm
C
D
E
F
A_{260}
0.9
1.0
A_{520}
0.09
0.06
A_{520}
0.5
0.4
0.3
A_{520}
2.0
1.5
1.0
40 60
40
60
80
T (°C)

Figure 39

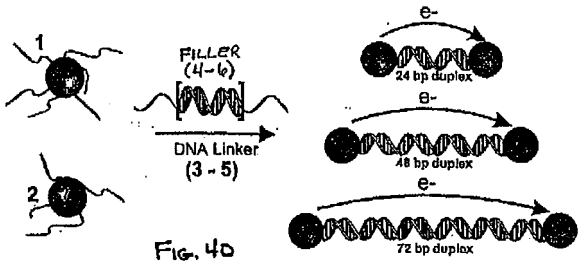

1
2
FILLER
(4-6)
DNA Linker
(3 - 5)
e-
24 bp duplex
e-
48 bp duplex
e-
72 bp duplex

FIG. 4D

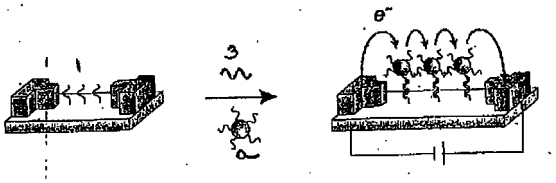
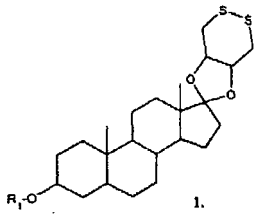

3
e-

FIG. 41

Fig. 42



1.

II $HS-(CH_2)_6OR_1$

III $S-(CH_2)_6OR_1$
$S-(CH_2)_6OH$

R_1

a = H
b = $(iPr)_2NP(OCH_2CH_2CN)$-
c1 = 5'-$p(A_{20})$-TATCGTTCCATCAGCT
c2 = 5'-$p(A_{20})$-TTGATCTTCCGTTCT
Target I = 79-mer oligonucleotide with target region:
3'- .....ATAGCAAGGTAGTCGAGCAACTAGAAAGGCAAGA.....5'

Fig. 43

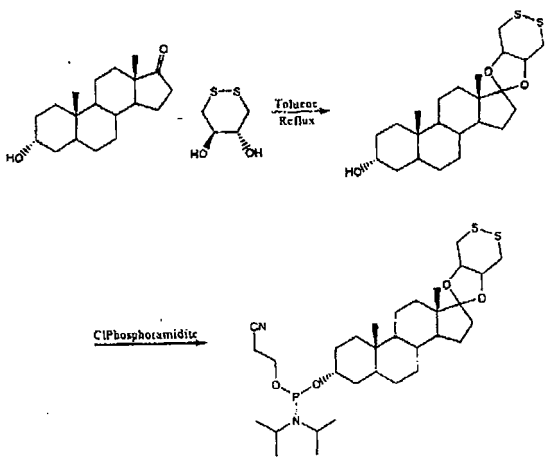
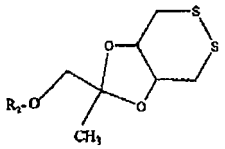


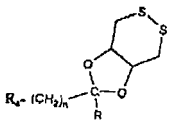
Fig. 44



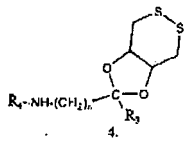
2.

R_2

a = H
b = $(iPr)_2NP(OCH_2CH_2CN)$-
c1 = 5'-p(A_{20})-GCAGACCTCA
c2 = 5'-p(A_{20})-CCTATGTGTCG
d = 5'-p(A_{20})
Target I – 63-mer oligonucleotide with target region:
3'.....CGTCTGGAGTGGATACACAGC.....5'



3.



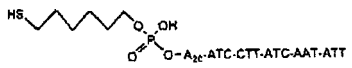
4.

R_3 = hydrogen, an alkyl group, an aryl group, or a substituted alkyl or aryl group

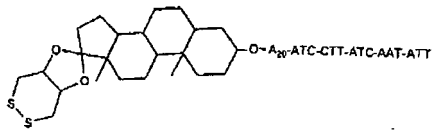
R_4 = an attached oligonucleotide or modified oligonucleotide

FIG. 45

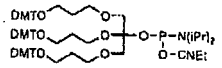
5.

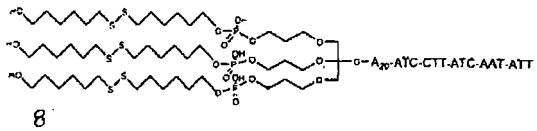

HS
OH
O-A20-ATC-CTT-ATC-AAT-ATT

6


O-A20-ATC-CTT-ATC-AAT-ATT

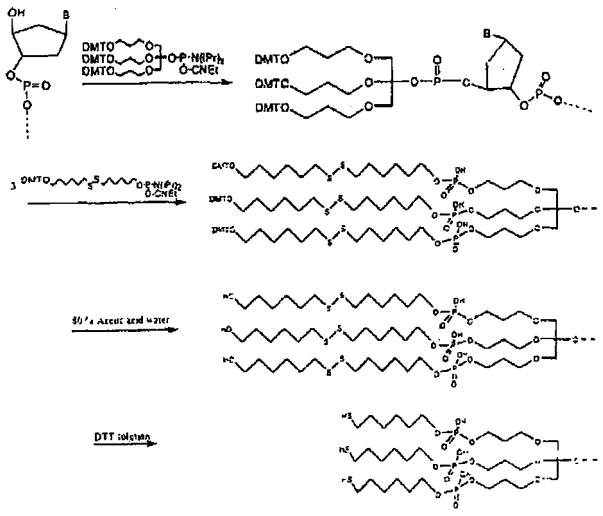
7


DMTO
DMTO
DMTO
O-P-N(iPr)2
O-CNE


O-A20-ATC-CTT-ATC-AAT-ATT

8

Fig. 46



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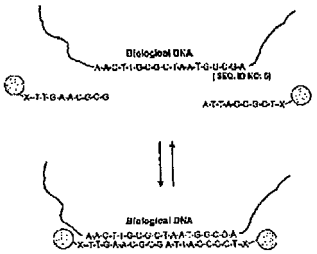
[Continued on next page]

(54) Title: NANOPARTICLES HAVING OLIGONUCLEOTIDES ATTACHED THERETO AND USES THEREFOR



(57) Abstract: The invention provides methods of detecting a nucleic acid. The methods comprise contacting the nucleic acid with one or more types of particles having oligonucleotides attached thereto. In one embodiment of the method, the oligonucleotides are attached to nanoparticles and have sequences complementary to portions of the sequence of the nucleic acid. A detectable change (preferably a color change) is brought about as a result of the hybridization of the oligonucleotides on the nanoparticles to the nucleic acid. The invention also provides compositions and kits comprising particles. The invention further provides methods of synthesizing unique nanoparticle-oligonucleotide conjugates, the conjugates produced by the methods, and methods of using the conjugates. In addition, the invention provides nanomaterials and nanostructures comprising nanoparticles and methods of nanofabrication utilizing nanoparticles. Finally, the invention provides a method of separating a selected nucleic acid from other nucleic acids.

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— *with international search report*

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【国際調査報告】

INTERNATIONAL SEARCH REPORT

Inte ...al Application No
PCT/US 01/01190

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68 C07H21/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, MEDLINE, CHEM ABS Data, BIOSIS, EMBASE, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 98 04740 A (UNIV NORTH-WESTERN ;MIRKIN CHAD A (US); MUCIC ROBERT C (US); E_GHAN) 5 February 1998 (1998-02-05)	
A	WO 99 60169 A (MOLECULAR MACHINES INC) 25 November 1999 (1999-11-25)	
A	WO 98 17317 A (SUEDDEUTSCHE KALKSTICKSTOFF ;BAYER ERNST (DE); FRITZ HANS (DE); MA) 30 April 1998 (1998-04-30)	
A	WO 93 25709 A (MEDICAL RES COUNCIL ;HAWKINS TREVOR LEONARD (US)) 23 December 1993 (1993-12-23)	
A	US 5 900 481 A (LOUGH DAVID M ET AL) 4 May 1999 (1999-05-04)	
	-/--	

[X] Further documents are listed in the continuation of box C. [X] Patent family members are listed in annex

* Special categories of cited documents:
'A' document defining the general state of the art which is not considered to be of particular relevance
'E' earlier document but published on or after the international filing date
'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
'O' document referring to an oral disclosure, use, exhibition or other means
'P' document published prior to the international filing date but later than the priority date claimed
'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
'&' document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 July 2002	12/08/2002
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Authorized officer Molina Galan, E

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 01/01190

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CHEMICAL ABSTRACTS, vol. 132, no. 4, 24 January 2000 (2000-01-24) Columbus, Ohio, US; abstract no. 3133C, LETSINGER, ROBERT L. ET AL: "Chemistry of oligonucleotide -gold nanoparticle conjugates" XP002205941 abstract & PHOSPHORUS, SULFUR AND SILICON AND THE RELATED ELEMENTS (1999), 144-146, 359-362	
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T	LI ZHI ET AL: "Multiple thiol-anchor capped DNA -gold nanoparticle conjugates." NUCLEIC ACIDS RESEARCH, (2002 APR 1) 30 (7) 1558-62. , XP002205940	

*Special categories of cited documents (see sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

National application No.
PCT/US 01/01190

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
Because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1-486 (partially)
Because they relate to parts of the international Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
Because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1998)

International Application No. PCT/US 01 01190

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-486 (partially)

In view of the large number of (independent) claims presently on file and their wording, which render it diff'cult, if not impossible, to determine the matter for which protection is sought, the present application fails to comply with the clarity and conc'seness requirements of Article 6 PCT (see also Rule 6.1(a) PCT) to such an extent that a meaningful search is impossible.

An effort was nevertheless made by the ISA to identify a main subject on which a meaningfull search could be performed. The subject apparently most important for the applicant is the preparation of oligonucleotide-nanoparticle conjugates using linkers comprising a steroid residue attached to a cyclic disulfide (cf page 34, line 18 to page 35, line 24). The resulting conjugates have, according to the applicant, an unexpectedly improved sensitivity and surprising stability. This was the only indication enabling the ISA to focus the search within the hundreds of possible embodiments disclosed in the application. The search has therefore been globally performed for the preparation of ligonucleotide-nanoparticle conjugates using linkers comprising a steroid residue attached to a cyclic disulfide, the resulting conjugates and their uses in nucleic acid detection.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int... nal Application No
PCT/US 01/01190

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
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			US	2002042112 A1	11-04-2002
			US	6133436 A	17-10-2000

Form PCT/ISA/210 (patent family annex) (July 1998)

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G01N 33/483	G01N 33/483	F	
G01N 33/566	G01N 33/566		
G01N 33/58	G01N 33/58	A	
G01N 37/00	G01N 37/00	102	
	C12N 15/00	ZNAA	
	C12N 15/00	F	

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(32)優先日 平成13年1月12日(2001.1.12)
(33)優先権主張国 米国(US)

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2G054 BB08 CA22 EA03 GA04
2G060 AD06 AE20 AF08 AG11 GA04 HC08 HC13 HC19 HC22 KA10
4B024 AA11 AA19 CA04 CA09 HA12
4B029 AA07 AA23 BB20 CC03 FA15
4B063 QA19 QA20 QQ08 QQ42 QQ52 QR08 QR42 QR56 QS25 QS34
QS39 QX02